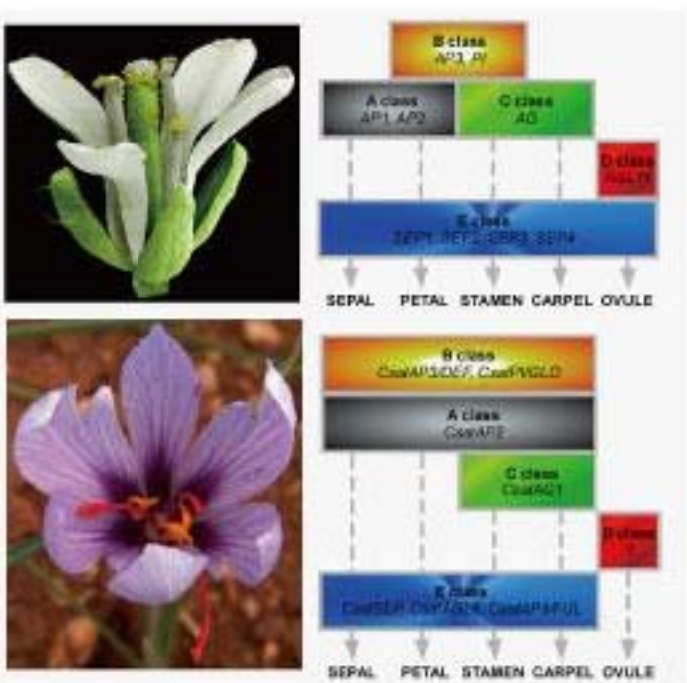
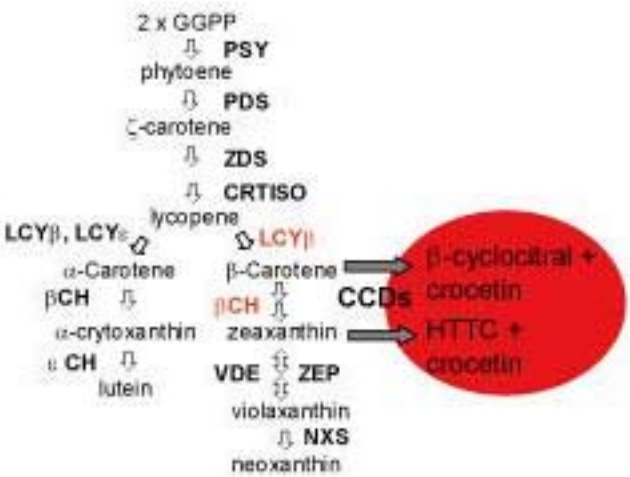
ISBN 978-4-903313-67-2

FUNCTIONAL PLANT SCIENCE & BIOTECHNOLOGY





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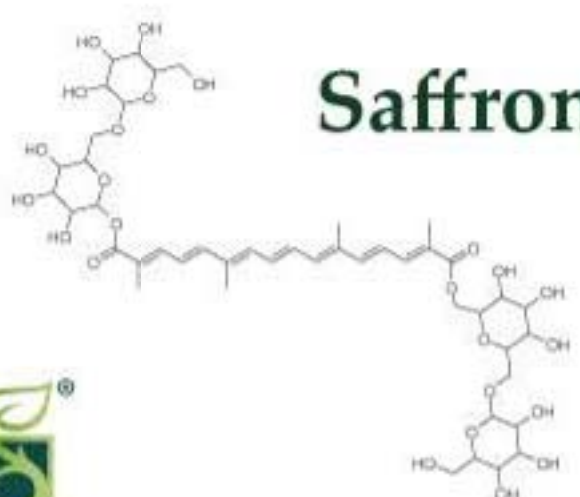
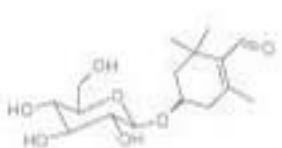


Editor
Amjad M. Husaini



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Global Science Books, Ltd.
Editorial Office
Miki cho Post Office, Kagawa ken, Kita gun
Miki cho, Ikenobe 3011-2, P.O. Box 7
761-0799, Japan

Head Office: Isleworth, United Kingdom
Accounting: Lagos, Portugal

GSB homepage: www.globalsciencebooks.info
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Editor

Dr. Amjad Masood Husaini

Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir, India

Cover photos/figures: Left: Classic ABC model vs modified ABC model for floral organ identity in *Crocus* (Tsaftaris *et al*., pp 38-44). Top and bottom right: Schematic carotenoid biosynthetic pathway in *Crocus sativus* (bottom right) stigma and main apocarotenoids generated (Gómez-Gómez *et al*., pp 56-63). Center right: *In vitro* response of corm slices of *Crocus sativus* L. (Quadri *et al*., pp 132-135). Bottom left structure: crocin. Bottom center structure: picrocrocin.

Printed in Japan on acid-free paper.
Published: December, 2010.

The Editor

Dr. Amjad Masood Husaini, a young Scientist working as Assistant Professor in Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir (India) holds a Ph.D. in Biotechnology and PG Diploma in Bioinformatics (Jamia Hamdard, New Delhi), besides certificates in Intellectual Property Rights (Indian Law Institute, New Delhi) and Remote Sensing Applications in Agriculture (Indian Agricultural Research Institute-Indian Space Research Organization). Recipient of Young Scientist Award-2009 in Agriculture (Jammu & Kashmir State Council for Science & Technology, Government of J&K), Jawahar Lal Nehru Award for Agricultural Research-2008 (Indian Council of Agricultural Research, Government of India), Junior Scientist of the Year Award-2007 (National Environmental Sciences Academy, New Delhi), he is listed among Top 100 Scientists of 2010 by the International Biographical Centre (IBC, Cambridge), and his biography included in 27th edition of Marquis Who's Who in the World. With an illustrious academic career Dr. Husaini holds the distinction of being top position holder in National Eligibility Tests for Life Sciences and Agricultural Biotechnology in India. His publications include book entitled 'Strawberry- Transgenics for stresses' and more than two dozen research/ review papers in National and International journals of repute, discussing different aspects of agricultural research and technology.

Dr. Husaini serves as member of professional associations like World Association of Young Scientists, New York Academy of Sciences, The Indian Science Congress Association, Biotechnology Society of India, National Environmental Science Academy (India), Young Professionals' Platform for Agricultural Research for Development, Scientists Without Borders, International Association of Computer Science and Information Technology, Royal Society of Crop Science, International Society for Biosafety Research, and serves in the capacity of editor/ associate editor etc. in editorial boards of various International journals of repute.

Foreword

Amjad Masood Husaini

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There is probably no other spice as evocative and fascinating as saffron. This is evident from its association with Greek gods, cultivation in hanging gardens of Babylonia, reference in Bible (Song of Solomon) for its essence and aroma, utilization for medicinal purposes by practitioners like Hippocrates and Pliny, and as cosmetics by women like Cleopatra. Even after centuries it remains tantalizingly exotic, awakening a gluttonous desire. Nothing more than the dried stigmas of fragile blossoms the autumn-flowering purple *Crocus* flowers are still gathered by hand from the ancient fields of Iran, Greece, Italy, Kashmir and Spain. Most saffron is grown in a belt of land ranging from the Mediterranean in the west to Kashmir in the east. Annually, around 200 tonnes of saffron are produced worldwide, and Iran, Spain, India, Greece, Azerbaijan, Morocco and Italy are the major producers of saffron. Its cultivation has even spread beyond these conventional boundaries to unconventional areas in countries like China, Afghanistan, France, Switzerland, Turkey, Azerbaijan, Japan, Australia (Tasmania), New Zealand, Argentina, and USA. In contrast there are some countries where its cultivation has almost disappeared (Germany, Austria and England), especially due to increasing labour costs. In order to reverse this trend and to reduce the cost of labor component new technologies need to be developed, disseminated and adopted. The present expectation of saffron producers and consumers from plant biologists is to make effort for its safe and sustainable production, avoiding as much as possible the use of chemicals, and make it convenient to get supply of pure un-adulterated high quality saffron freely to the end-users. With the scope of breeding programs being limited, the implementation of biotechnological approaches can be helpful in attaining these goals. Besides, saffron can be adopted as a model crop for molecular and physiological studies of spices, like enhanced odour, taste, colour and aroma. The aim of this compendium, is to provide updated information about origin of saffron, its medicinal properties, flower development, scope of –omics approaches in saffron improvement, state of current understanding about aroma/colour development in saffron etc., through the inclusion of chapters written by leader groups in these areas.

Based on the analysis of archaeological, historical, botanical, cytological, geographic, molecular and reproductive biology of saffron and allied species, Caiola and Canini have made an excellent attempt to establish its site and nature of origin. Though the period of its origin is still disputed, but it is widely accepted as to have originated in pre-Hellenic and Hellenic periods. Sharma and Piqueras discuss the application of tissue culture for micropropagation and secondary metabolite production, in order to meet the ever increasing demand of saffron. They argue that tissue culture techniques offer the capability to produce large quantities of propagating material in short duration of time, plus production of commercially important chemical constituents like, crocin, picrocrocin, crocetin and safranal under *in vitro*. However, the protocols available so far need further refinement for their commercial utilization. Research shared on a successful attempt by Quadri *et al*. to induce cormogenesis and maximize corm size under in vitro is a nice supplement. Fiore et al. discuss the role of –omics approaches as effective tools in saffron investigation and argue that until the entire genome of saffron is totally sequenced, or all the transcripts preferentially expressed during each stage of stigma development characterized, we are still far from an accurate elucidation on the genetic features of this important spice. Husaini and Ashraf stress on the integration of these –omics approaches to dissect the molecular basis of flavour and colour biogenesis, genomic organization and biology of gynoecium. Since such an approach would lead to generation of huge data sets whose interpretation become imperative, therefore, role of bioinformatics for ‘extracting’ such useful information has been discussed at length.

Understanding the mechanism of flower development in saffron is central to saffron improvement. Tsaftaris *et al*. have highlighted the role of *Crocus* MADS box genes and discussed their expression patterns in leaves and the four flower organs: outer tepals, inner tepals, stamens and carpels. The expression analysis of these genes support the hypothesis that a modified ABCDE model in the flower of *Crocus* is responsible for the development of the different *Crocus* flower organs and the transformation of the sepals and petals into tepaloid organs, designated outer tepals and inner tepals, respectively. Understanding the pathways that contribute to aroma and colour formation is of prime importance for metabolic engineering of saffron. Even though the enzymes involved in the generation of the main saffron apocarotenoids remain at the moment elusive, Gómez-Gómez *et al*. provide a comprehensive picture of the molecular regulation of colour and flavour biosynthesis in *C. sativus* in their mini-review. Saffron purity is a major concern for the consumers/dealers; and picrocrocin, being exclusive to saffron only, acts as an excellent marker of saffron purity. Maggi *et al*. have reviewed the importance of accurate determination of picrocrocin and summarized the available methodologies, pointing out the gaps contained in the current ISO 3632 Standard, normally used in the international market.

Hundreds of research papers have been published related to the biological properties of saffron. Premkumar and Ramesh have made an attempt to provide an overall gist of these properties, laying particular emphasis on anticancer, antimutagenic and antioxidant potential, while a research study contributed by Neamati and Boskabady presents results on the effect of saffron, especially safranal on muscarinic receptors on tracheal chains in guinea pigs. Since most of the research published about antioxidant properties, cancer, tumor, neuronal injury and sedative effect, etc of saffron makes use of animal models; use of such animal models causes difficulty in understanding its exact role in human application. In the review by Licón *et al*. a novel attempt has been carried out to translate animal doses to human intake when saffron is included on the diet, in order to make an estimation of the potential healthy effects in humans. In contrast, Gómez-Gómez *et al*. provide an overview of the important spices responsible for allergic reactions, with special attention given to saffron. In an interesting article Botsoglou *et al*. discuss the scientific data on the biological properties of saffron, and investigate its possible use as a feed additive in poultry industry. The industry would certainly appreciate such replacement of synthetic antioxidants in place of natural ones, and thus satisfy consumer demands for production of eggs and meat without residues from substances having potential harmful affect on human health.

Saffron plants are adversely affected by a multitude of pathogenic organisms including bacteria, fungi, viruses and nematodes. Plants use sophisticated defensive strategies to resist their invasion, using both preformed and inducible defence responses. These saffron pathogens and the defence responses have been discussed in the chapter of Ahrazem *et al*. emphasizing the role of soil conditions, drainage, temperature, and farming practices like planting date and application of fertilisers or herbicides. Finally, Husaini *et al*., while discussing various production practices followed by saffron growers of Kashmir highlights the current problems faced by saffron industry in India. The book concludes with Husaini et al. proposing some technological interventions like using breeding, *in vitro* cormlet and stigma production, and mechanization for sustainable saffron production. Further, policy interventions for revival of saffron industry in Kashmir are also suggested.

It is my hope that the saffron scientists will find this concise book helpful in moving forward in their important quest of contributing in the interest of saffron producers and consumers. I would like to thank Dr. Jaime A. Teixeira da Silva and Kasumi Shima at Global Science Books Ltd., for their cooperation and helpful suggestions; and my family for their understanding and support during the prolonged and time-consuming work on this volume.

December, 2010

Foreword

Prof. A. R. Trag

Director of Research

Sher-e-Kashmir University of Agricultural Sciences &
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Saffron occupies an important position in the agricultural scenario of Jammu & Kashmir state of India), and holds an equally significant place in the economies of countries like Iran, Spain, Italy and Greece etc. The production and area under this crop showed a declining rend in Jammu & Kashmir till recent past, due to lack of R&D support. SKUAST-K and other research institutes have developed relevant technologies for boosting saffron production and demonstrated these to saffron growers. With the adoption of new technologies and implementation of projects, the saffron production is again showing increasing trend in J&K state. However, the adoption of improved cultivation practices and post-harvest technologies needs to be pursued more vigorously by extension agencies and scientists. As such the publication of a Special Issue on Saffron in 'Functional Plant Science and Biotechnology' by Global Science Books, U.K. is a welcome step. I wish to congratulate the editor, Dr. Amjad Masood Husaini, Assistant Professor, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir, India, and all other distinguished Scientists from various Research Institutes across the globe for contributing their valuable articles and making the publication of this valuable Edited Book a possibility. I am sure that this book would be a handy document for use by saffron researchers, students, growers and traders.

November, 2010

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Foreword

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Saffron (*Crocus sativus*) which belongs to Iris family *Iridaceae* is the most expensive spice in the world and is popularly known as the "Golden Condiment". In India it is a legendary crop of Jammu and Kashmir, produced on well drained karewa soils where ideal climatic conditions are available for good shoot growth and flower production. Iran, Spain and India are the major saffron-producing countries in world with Iran occupying the maximum area, contributing about 85% to the World's saffron production. In the recent past a consortium, composed of 14 partners of EU and non-EU countries has taken the responsibility of creating and maintaining the genetic variability of saffron and the European Commission has approved a project on "Genetic Resources of Saffron and Allies (*Crocus* spp.): CROCUSBANK". In India we need to increase production by bringing more area under its cultivation and double the average productivity to make it globally competitive and remunerative to growers. In view of this a National mission on saffron was launched recently by the Government of India to ensure the revival of saffron production in Jammu and Kashmir. In 2010 the Central Government approved a plan to release Rs. 3.76 billion under this National Saffron Mission Programme for four years with the aim of improving overall saffron production, enhance its quality, build research and extension capability and develop an appropriate system for organized marketing. Premier research institutions like Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir and Central Institute of Temperate Horticulture are involved in the mission, while Development Departments of Jammu & Kashmir government are also key coordinators. Besides, many projects at these research institutions are funded under the Horticulture Technology Mission. The Indian Council of Agricultural Research is the nodal agency implementing these programs and the major thrust is on saffron corm multiplication, quality corm production, *in vitro* production of

saffron/microcorms, corm rot management, saffron inter-culture, and demonstration of developed technologies to saffron growers. The Department of Biotechnology is also funding projects of individual researchers for development and refinement of saffron production and improvement technologies with a particular thrust on *in vitro* cormlet production and production of stigma-like structures.

The present document is a commendable effort in presenting some important studies on saffron with emphasis on modern scientific approaches, which I believe would surely help realize the above discussed priority objectives. I compliment the editor Dr. Amjad Masood Husaini, Scientist, SKUAST-K (India) for bringing out such a relevant compilation of this special unique on Saffron, in which eminent scientists across the globe have contributed their valuable research and papers. Moreover, credit needs to be given to Global Science Books (GSB), UK for their efforts in publishing such an important compendium. I hope this volume would bring to the forefront a number of technical, socioeconomic and policy issues useful in guiding saffron biotechnologists, botanists, cultivators and policy makers.

December, 2010

CONTENTS

How to reference: *Caiola MG, Canini A (2010) Looking for Saffron's (Crocus sativus L.) Parents. In: Husaini AM (Ed) Saffron. Global Science Books, UK, pp. 1-14*

Looking for Saffron's (*Crocus sativus* L.) Parents

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ABSTRACT

The authors analyze the archeological, historical, botanical, cytological, geographic, molecular and reproductive biology of saffron and allied species in order to establish its site and parent origin. The authors have studied saffron, *Crocus sativus* and the diploid species *C. cartwrightianus*, *C. thomasii*, *C. hadriaticus* and compared them with what was previously known from the literature. When saffron originated is still open to dispute. It has been widely known since the pre-Hellenic and Hellenic periods, but it is impossible to detect if was *C. sativus* or other *Crocus* species such as *C. cartwrightianus.* Concerning the site origin the research indicates two possible sites: one in Greece in the Mediterranean area, the other at East in Turkey-Iran-India. In both areas, records and place names connected with various species of *Crocus* constitute an important information source for the presence of saffron. Cytological, DNA, and reproductive studies on the allied species of *C. sativus* such as *C. cartwrightianus*, *C. thomasii*, *C. hadriaticus*, indicate a more likely parent of saffron may be *C. cartwrightianus* or *C. thomasii*. Both these species are diploid with a karyotype similar to saffron. In addition, their pollen can fertilize the egg cell of saffron, giving rise to seeds which are viable, germinate and form new corms. Thus, saffron can originate through fertilization of a normal reduced egg cell with an unreduced male gamete of the same *Crocus* species or by crossing between an egg cell and the male unreduced gamete of another species. The origin of Saffron by allopolyploidy seems more probable considering the recent data on its karyotype and molecular biology.

Keywords: *Crocus cartwrightianus*, *C. thomasii*, *C. hadriaticus*, progenitor/s of saffron

CONTENTS

INTRODUCTION

Crocus sativus L. (Iridaceae) is a small geophyte, cultivated worldwide and known as a source of the spice saffron that is used for cooking, staining, medicine, cosmetics and some other purposes. Saffron is obtained from the dried stigmas of flowers and marketed as saffron filaments or as powder from milled stigmas. It is the most expensive spice. Saffron cultivation areas as well as the produced amount have decreased worldwide, particularly last century, due to high costs for the production of the spice. By contrast the use of and demand for saffron have increased in recent years due to the promotion of new products containing saffron and to improvement in the spice's quality and its conditions of cultivation (Winterhalter and Straubinger 2000; Fernández 2004, 2007).

The interest in saffron studies and application has been reported in three symposia on Saffron Biology and Technology held respectively in Spain (2003), in Iran (2006), in Greece (2009). The research presented and discussed in these symposia forms a basis for the guidelines for the management of saffron plant and for amelioration programmes based on vegetative or crossing reproduction. The increase in grown saffron is related to the possibility of obtaining higher yields per hectare and the high quality of commercial saffron. The solution to these two problems needs genetic amelioration of the plant, the amelioration of cultivation practices, as well as controlled conditions during the preparation, storage and marketing of the spice. Successful experiments based on the clonal selection of the more productive corms of saffron (Agayev *et al*. 2009) have recently been carried out. Extensive labour will result in higher yield production in future years with the consequence that saffron growth and production will increase. The genetic amelioration of saffron by crossing requires knowledge of the original species or wild relatives as well

as of the ways and site of origin of the cultivated plant. Many studies have been dedicated to solving this puzzle although with disputed results, the exact parents of saffron remaining an open question.

Most of these studies centre round the question as to whether saffron is a true species or whether it should be considered a triploid mutation derived from a wild species by autotriploidy (Chichiriccò 1984; Mathew 1982, 1999; Negbi and Negbi 2002). According to Negbi and Negbi (2002) saffron parent should be *C. cartwrigtianus* and the Aegean islands site origin where this species occurs and is still used today by inhabitants. Alternatively it is the result of allotriploidy obtained by crossing process between two wild species (Feinbrun 1958; Brighton 1977; Agayev 2002; Castillo *et al.* 2005). In the first case research is engaged in detecting the living or remote wild saffron progenitor. Alternatively we may have to search for more than one possible progenitor (Grilli Caiola 2006). In all these cases we have to establish the possible site of origin of the progenitor by comparing saffron to species which are genetically and geographically close to *Crocus sativus*. Such work needs the cooperation of many disciplines such as archeology, classic literature, botany, cytology, genetics, phytogeography, biochemistry, molecular biology.

Thanks to the work produced in various saffron-growing countries, a large amount of information has been accumulated on saffron biology and cultivation. Here we will review these studies on saffron parentage and origin by considering studies on archaeological and historical records as well those on cytological, systematics, geographic distribution and utilization of saffron and allied *Crocus* species.

HISTORICAL BACKGROUND

The archaeological and historical records are important for establishing the presence of saffron in a area as well for tracking its spread elsewhere and, finally, for arriving at a conclusion about the possible progenitor of saffron present in the same areas.

The presence of saffron is very ancient in the Mediterranean area as is testified by records, pictures, written throughout the region.

Saffron is a very charm fascinating plant, the long history of which fluctuates between myth, legend and history. According to the mythology Krokos was a beautiful boy in love with the nymph Smilax. At least until Hera, out of jealousy, transformed Krokos into the flower *Crocus* and Smilax into the climbing plant *Smilax* (Cattabiani 1996).

According to a legend the Greek god Zeus slept on a bed of saffron. Another legend relates how Alexander The Great, during the campaign to the East, when he reached Kashmir, settled his army on a plain. The following morning he saw an ocean of violet flowers around his tents and under the hooves of his horses. The flowers could only have been saffron (Aucante 2000).

Archaeological studies carried out on Crete, Cyclads Islands, and mainland Greece have revealed the presence of saffron in all these areas. *Crocus* was known in the Minoan period in Crete three thousand years ago. On the wall of the Minos Palace, at Knossos (Crete), (1700-1600 B.C.) are frescoes depicting crocus-gatherers. *Crocus* flowers are also observable on the skirts and belts of small statues and other objects found in the same Palace (Chirassi 1968). Over 150 bossom bowls were widely distributed in both settlements and tombs throughout Crete, the Aegean and Byblos and Troy. These vessels were more popular during the Neopalatial period (1700-1425 BC) and were made of serpentine carved with a decoration of six broad petals. Such a design suggests a flower of saffron *Crocus* commonly depicted in Cretan wall paintings (Bevan 2007). Other important records are found in the Palace of Akrotiri in Thera (now Santorini) where frescoes represent young women collecting crocuses (**Fig. 1**) and offering them to a divinity (Douskos 1980; Marinatos 1984; Amigues 1988; Nugent 2009). Archeologists interpret the depicted flowers as crocus saffron

Fig. 1 Gatherers of Crocuses on Wall paintings from Xeste 3 building, Akrotiri, Thera. With kind permission from the Thera Foundation – Petros M. Nomikos.

used in ritual ceremonies in the pre-Hellenic and Hellenic ages, in medicine (Forsyth 2000; Ferrence and Bendersky 2004) and as fine dye (Sarpaki 2000). Interesting is the contribution by Sarpaki (2000) on the way of collecting, preparing and using wild *C. cartwrightianus* in Thera in the past. They are similar to those adopted today in the same Island. In addiction any other *Crocus* was grown during Minoan and Cycladean era. Miniature frescos of the seventeenth century B.C. representing saffron flowers were found in Syria-Palestina, suggesting that this plant was known and used in the Near East (Niemeier and Niemeier 2000).

Saffron-based pigments have been found in 50,000 year-old depiction of prehistoric beasts in the region that now is Iraq. Later Sumerians used wild growing saffron in their remedies and magical potions (Willard 2001). Ancient Persian cultivated Persian saffron *C. sativus hausknechtii* in Derbena, Khorasan by the Xth century B.C.

Toponimy suggests the presence of saffron in sites such as Krocos at Kozani in Greece and in Safranbolu (Turkey), the town chosen as a World Heritage City by United National Educational Scientific and Cultural Organization (UNESCO) in 1994 due to its well-preserved Ottoman houses and architecture. This is a site where saffron is grown and every year around the city the Festival of Harvest Saffron is celebrated. Another site is the unknown town Azupirano, "The City of Saffron", on the banks of river Euphrates, Iran. It is the city where Sargon, founder of Akkadian Empire, is born about 2300 BC. By 1000 B.C. saffron was being widely used in Iran where it was a symbol of love and luxury (Basker and Negbi 1983). In the 4th century B.C. a main cultivation area of saffron was Corycos in Cilicia, the Mediterranean coast of Turkey. It is probable that the name of Corycos derives from *Crocus*. Other toponimy is Saffron-Walden in England.

Many Greek, Roman, and Egyptian historians, reported the use of saffron as a precious component to stain cloth, to give special colour and taste to food and drink, and for use in medical therapy (Basker and Negbi 1983; Tammaro 1987; Porter 2000). Authors such as Aeskylus (525-456 B.C.), Sophocles (497-406), Hippocrates (460-370), Aristophanes (445-384 B.C), Theophrastus (371-287 B.C), Plautus (255-184B.C.), Varro (116-27 B.C.), Celsus (14-37 A.C.), Pliny the Elder (23-79 A.C.), Galen (129-200 A.C.), and Petronius (?-66 A.C.) cited the use of crocus in their works. In "Oedipus at Colonus" Sophocles wrote that *"the bodies of Demetra and Core were embellished by a crown of Narcissus and Crocus"*. Aeschylus in *"Persea*" describes

the Queen invocating her husband King Dario with *"shoes dyed with Crocos"*. Plautus (255-184 B.C.) in "*Aulularia*" mentions *"the dyers of crocos"*.

From the above reported information it turns out that use of crocus was widespread and perhaps grown in the past. Greece is today the homeland of around 40% of the world's wild *Crocus* diversity (Tsoktouridis *et al*. 2009). It is possible that among the numerous species, saffron also originated. However the word "Crocus," or "Krocos" does not guarantee the identification of the crocus flower with saffron, the plant nowadays used and cultivated. In fact it is possible that other *Crocus* species could be used for similar purposes. At Santorini, in the Cyclads Isles (Greece) *Crocus cartwrightianus* occurs as a wild plant which the local habitants use as saffron.

Theophrastus (*Historia plantarum*) and Pliny (*Naturalis Historia*) described many types of crocus. Theophrastus said "In autumn bloom the crocus, both the scentless mountain form and the cultivated one (saffron-crocus); for these bloom directly the first rains come; crocus is reproduced by roots". Pliny wrote: "Among the crocum types the wild is the best but it is not convenient for growing in Italy in that it reduces its growth. Crocum cultivated is larger, and more fine but more delicate; it easily degenerates and does not produce much. The most appreciated crocum is from Cilicia, mainly that from Mount Corico, then that from Licia of Mount Olympus, and after that of Sicily. According to some authors second place for saffron quality goes to crocus from Thera". Varro (De re rustica) suggests "sowing lilium and crocum" during the period of Pleiads, around October". Similar suggestions occur in "Res Rustica" of Columella (second century A.C).

From the above reported citations it is possible to deduce that the Mediterranean region is one of the probable sites of saffron origin; another site could being in the East, in Turkey-Iran- India, where saffron cultivation is reported to be thousands of years old. According to some Authors (Alberini 1990; Winterhalter and Straubinger 2000) saffron originated at first in Iran and Kashmir from where Phoenicians introduced it to the Greek and Roman world. Later (about 960 A.C.) it was brought by the Arabs and Moors to Spain. In effect the term in ancient Greek is "koricos"; the Roman's used the term "crocum"; by contrast saffron probably originates from the Arabic word Zafaran, zaafar (Gerarde 1636). The Arabic "safran" is quite similar in various other languages: English, saffron; Italian, zafferano; French, safran; Spanish, azafran; German, saffran; Russian, shafran; Turkish, zaferen. This consideration suggests how ancient is its use worldwide.

In Europe, the diffusion of saffron was thanks to the Arabs who brought it to Spain and other territories such as Sicily. In France, it appears during the Crusades at the end of the 1300s A. D. In Provence, the major development of saffron occurred in XI and XVII centuries. In Great Britain, it was introduced in XIV century from Kashmir and then from Tripoli in 1582. It was grown in Saffron-Walden in Essex for colouring and medicine, but others sites of saffron cultivation such as Cambridge are reported (Gerarde 1636).

In Italy, saffron cultivation was introduced between 800 and 900 A.D., at first in Sicily, then in Calabria, Umbria, Tuscany and Abruzzo (about 1400 A.D.). Navelli, L'Aquila, for many centuries has been the main saffron cultivation centre in Europe, till at least to the 1960s and 70s when the production cost caused a decrease in the plant's cultivation. Recently, after appropriate cooperative organization the saffron grown has increased in Abruzzo and been extended to many other Italian regions including Tuscany, Liguria, Umbria, Sicily, Calabria, and Sardinia (Gresta *et al.* 2007).

MORPHOLOGY AND SYSTEMATICS OF *CROCUS SATIVUS* AND ALLIED SPECIES

Until Linnaeus saffron was referred to as "cultivated crocus". It was known for its morphology, infertility and uses, being distinct from wild crocus mainly on the basis of morphological, flowering period and growing characteristics. The name Saffron was used by Gerarde in 1597, when the first edition of his "The Herball or Generall Histories of Plants" appeared. This Author in the edition of 1636 described cultivated saffron, *C. sativus* and other wild spring flowering saffron afterwards identified as *C. flavus*, *C. vernus*, *C. versicolor*, *C. nudiflorum*.

The scientific name of saffron, is due to Linneaus who in 1762 named *Crocus sativus* var. *officinalis*, a cultivated *Crocus* of Family Iridaceae. The species *Crocus sativus* L. is now recognized by Mathew (1982, 1999; Frello *et al.* 2004). The systematics and taxonomy of *C. sativus* has been complicated by synonyms appeared in the literature as *C. sativus* var. *cashmirianus* Royle (1836); *C. orsini* Parl. (1858); *C. sativus* var. *orsini* (Parl.) Maw (1886). Until to arrive to Paradies (1957) who considered *C. thomasii* a geographical subspecies of *C. sativus* L. Then, Tutin *et al*. (1968) in *Flora europaea* described the species *C. sativus* as *C. cartwrightianus*, whereas Pignatti (1982) in *Flora d'Italia* reports *C. sativus* in *C. thomasii* Ten. and as *C. medius* Balbis.

However, many other *Crocus* have been found in the wild and cultivated for ornamental purposes. This induced some authors to give a systematic order to the numerous recognized species and intraspespecific taxa within the *Crocus* genus, taking into account the flowering period and the plant's morphological and geographical features (Herbert 1847; Maw 1886).

In the "*Crocus*" 1982 edition Mathew enumerated 80 species, of which 6 were identified in 1700, 54 in 1800, 20 in 1900. Recently Petérsen *et al.* (2008) listed 88 species, 8 new species being added to the previous ones from 1983 to 2007. According to Nørbaek *et al.* (2002) more than 100 cultivars of *Crocus* are known today, these being selected by means of hybridisation between relatively few species. Probably the list of *Crocus* species is far from closed and new taxa will be added in the future.

Mathew (1982, 1999) distinguished the *Crocus* genus into subgenus *Crocus* and *Crociris*. In subgenus *Crocus* are the sections: A) *Crocus*; B) *Nudiscapus*. The morphology of the corms, tunics, bracts, bracteols, leaves, flowers and seeds, the flowering period, cytological and ecological features have been used by Mathew to divide the *Crocus* genus into a hierarchy of sub-genus, sections and series and to define the species within those infrageneric groupings.

Anthers with extrorse dehiscence characterize the subgenus *Crocus*. Scapes subtended by a membranous prophyll, enclosed and ridden within the sheathing leaves or cataphylls distinguish the *section Crocus*. Corm tunics finely fibrous usually reticulatae; flowers autumnal, leaves rather numerous usually 5-30, appearing with the flowers or shortly after; bracts flaccid, usually not closely sheathing the perianth tube membranous, white or transparent with no markings; anthers yellow; style branches 3, usually and often expanded at the apex, entire or not at most fimbriatae; seed coats covered with a dense mat of papillae. All these features characterize the series Crocus. To series *Crocus* belong: *C. sativus*, *C. asumaniae*, *C. oreocreticus*, *C. moabiticus*, *C. cartwrightianus*, *C. mathewii*, *C. hadriaticus*, *C. thomasii* and *C. naqabensis*.

C. sativus L. (1762) according to Mathew 1982, 1999, (**Fig. 2A**) is "a geophyte with depressed-globose corms, flattened at the base, with fibrous tunics, finely reticolate, extending at the apex of the corm into a neck up 5 cm long (**Table 1**). Cataphylls are membranous, the leaves are usually synanthous, erect, green, glabrous or ciliate. Flowers are fragrant, deep-lilac purple, with darker veins and a darker violet stain in the throat which is white or lilac, and pubescent. Perigonium tube is with segments subequal. Stamens are purplich, glabrous and anthers exceeding at least half the length of the perianth. Perigonium segments arise at a point below the base of the anthers in the throat of the flower. Capsules and seeds have been only rarely reported, saffron being considered a sterile species. It is known only as a cultivated plant".

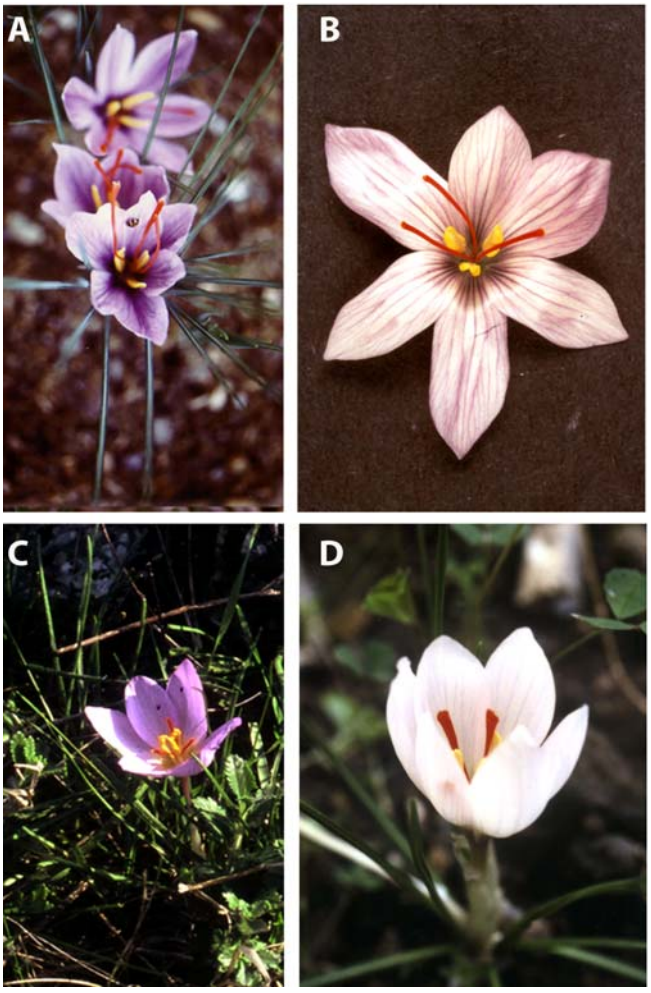

Fig. 2 Flowers of *Crocus* (**A**) *C. sativus*; (**B**) *C. cartwrightianus*; (**C**) *C. thomasii*; (**D**) *C. hadriaticus*.

C. cartwrightianus Herbert (1843) (Herbert 1847), (**Fig. 2B**). Description by Mathew (1982, 1999) is: "depressed-globose corms, with tunics fibrous, are finely reticulated, extended at the apex of the corms. Fragrant flowers are strongly veined darker, sometimes stained darker at the base of the segments and on the tube, sometimes pure white with no veining in the albinois. Throat white or lilac, pubescent. Perigonium tube is oblanceolate or obovate. Stamens are glabrous or slightly papillose at the base and anthers are yellow. Style is equaling or exceeding the anthers and at least half the length of the perigonium segments, arising at a point well below the base of the anthers and usually in the throat of the flower. Ellipsoid capsule raised on a pedicel above the ground level at maturity. It produces seeds (**Table 1**). It is known wild and cultivated plant".

C. thomasii Tenore (1826) (**Fig. 2C**). Description by Mathew (1982, 1999) is: "depressed globose corms with fibrous finely reticulated tunics extended at the apex of the corms into a neck up to 1 cm long. Synanthous leaves, usually equaling the flower at the anthesis, but sometimes only the tips showing, are green, glabrous or papillose at the margins. Fragrant flowers, generally not strongly veined are darker but sometimes veined or, stained violet towards the base of the segments; throat pale yellow, pubescent. Perigonium is elliptic, obovate or oblanceolate, acute or obtuse. Filaments usually pale yellow are glabrous or finely pubescent at the base; anthers yellow. Style divided at a variable point, usually ranging from just below or level with the base of the anthers to about a quarter of the way up the anthers, into 3 bright red branches, half or less than the length of the perigonium segments, expanded gradually to the apex. Capsules ellipsoid raised on a pedicel above ground level at maturity; seeds are globose, with poorly developed raphe and pointed caruncle (**Table 1**). It is known wild and cultivated plant".

C. hadriaticus Herbert (1845) (Herbert 1847) (**Fig. 2D**). Description by Mathew (1982, 1999) is: "Corms depressed – globose with tunics fibrous finely reticulated extended at the apex of the corm into a short neck. Cataphylls white, membranous. Leaves are normally synanthous, sometimes equaling the flower at the anthesis but sometimes very short and occasionally absent, but then appearing immediately after the flowers, grey-green, ciliate. Fragrant flowers are often stained externally brownish, yellowish or violet at the base of the segments; throat yellow or rarely white, pubescent. Perigonium tube is white, yellow, brownish or violet; segments equal or the inner slightly smaller, elliptic-oblanceolate, obtuse. Filaments yellow or white, glabrous or sparsely and minutely pubescent just at the base; anthers, yellow. Style divided into 3 slender branches, each branch slightly shorter than or exceeding the anthers, less than half the length of the perigonium segments, arising at a point above the throat of the flower. Capsule are ellipsoid and raised on a pedicel above ground level at maturity; seeds are reddish-brown, sub-globose" (**Table 1**).

C. moabiticus Bornmüller & Dinsmore ex Bornmüller (1912) (Kerndorff 1988). Description by Mathew (1982, 1999) is: "corms sub-globose with fibrous tunics parallel at the base and weakly reticulate at the apex extending into a neck. Cataphylls are white, membranous. Leaves are usually present but short at flowering time, grey-green, sparsely papillose on the margin of the keel. Flowers are fragrant, veined purple to varying degrees on all six segments on a white ground colour, sometimes so heavily as to appear purple, sometimes stained darker at the base of the segments and on the tube; throat white or purple, pubescent. Perigonium tube white or purple; segments sub-equal,

Table 1 Features of *Crocus sativus* and allied species.

	Cs[a]	Cc[b]	Ct[c]	Ch[d]	Cm[e]	Co[f]	Ca[g]	Cma[h]
corm (mm)	50 ± 1.0	12 ± 2.0	10 ± 2.0	12 ± 4.0	23 ± 8.0	12 ± 2.0	12 ± 2.0	16 ± 1.0
neck (mm)	50 ± 1.2	27 ± 3.0	15 ± 3.0	8.0 ± 1.0	70 ± 10	-	12 ± 1.0	19 ± 2.0
leaves (number)	8.0 ± 1.0	9.0 ± 1.0	8.0 ± 2.0	7.0 ± 2.0	19 ± 6.0	12 ± 2.0	5.0 ± 1.0	7.0 ± 1.0
flowers (number)	3.0 ± 1.0	3.0 ± 0.5	2.0 ± 1.0	2.0 ± 1.0	5.0 ± 2.0	2.0 ± 1.0	2.0 ± 1.0	2.0 ± 0.5
perigonium l. (cm)	4.0 ± 1.0	3.0 ±1.0	4.0 ± 2.0	6.0 ± 3.0	4.0 ± 2.0	4.5 ± 1.0	6.0 ± 0.5	7.0 ± 1.0
p. segments l. (cm)	4.0 ± 1.0	1.4 ± 0.5	2.0 ± 1.0	2.0 ± 1.0	1.5 ± 1.0	1.4 ± 1.0	2.5 ± 1.0 x	1.9 ± 1.0 x
	x 1.5 ± 1.0	x 3.2 ± 1.0	x 4.5 ± 2.0	x 4.5 ± 1.0	x 3.2 ± 2.0	x 3.3 ± 0.5	3.0 ± 1.0	3.0 ± 1.0
stamens: filaments length (mm)	1.0 ± 0.5	5.0 ± 1.0	6.0 ± 2.0	7.0 ± 4.0	2.5 ± 2.0	6.0 ± 2.0	4.0 ± 1.0	3.5 ± 1.0
stamens: anthers length (mm)	20 ± 5.0	10 ± 1.0	12 ± 2.0	9.0 ± 2.0	13 ± 4.0	13 ± 2.0	15 ± 3.0	11± 1.0
stigmas length (mm)	24 ± 2.0	16 ± 5.0	13 ± 2.0	13 ± 3.0	18 ± 7.0	17 ± 3.0	16 ± 2.0	-
capsule length (cm)	1.90 ± 1.0	2.0 ± 1.0	1.5 ± 0.5	1.5 ± 0.5	1.5 ± 1.0	1.5 ± 2.0	1.9 ± 1.0	2.0 ± 0.5
	x 1.40 ± 0.2	x 1.2 ± 1.0	x 0.6 ± 0.2	x 0.7 ± 0.1	x 2.5 ± 1.0	x 7.0 ± 0.5		
pedicel length	4.0 ± 1.0	4.0 ±1.0	3.5 ± 2.0	4.0 ± 1.0	very short	at ground level	n.d.	short
seed diameter (mm)	4.4 ± 0.2	3.4 ± 1.0	3.0 ± 1.0	2.0 ± 1.0	3.5 ± 1.0	3.0 ± 2.0	2.5 ± 1.0	4.0 ± 1.0
	x 3.2 ± 0.6	x 2.3 ± 0.5	x 1.8 ± 1.0	x 3.0 ± 2.0	x 3.0 ± 1.0			
flowering	Oct-Nov	Oct-Dec	Oct-Nov	Sept-Nov	Nov-Dec	Oct-Dec	Oct-Nov	Oct-Nov

[a]*Crocus sativus*; [b]*C. cartwrightianus*; [c]*C. thomasii*; [d]*C. hadriaticus*; [e] *C. moabiticus*; [f] *C. oreocreticus*; [g]*C. asumaniae*; [h]*C. mathewii*.; n.d.= not detected

narrowly elliptic to oblanceolate or obovate, acute to obtuse. Filaments are white ageing to purple, glabrous; anthers are yellow. Style divided into 3 deep red clavate branches, equaling to/or much exceeding the anthers and at least half the length perigonium segments, arising at a point well below the base of the anthers in the throat of the flower. Capsule are ellipsoid, carried on a very short pedicel at maturity, sometimes not exceeding the ground level; seeds dark brown, irregularly sub globose" (**Table 1**).

Crocus oreocreticus B. L. Burtt (1949) (Burtt 1948). Description by Mathew (1982, 1999) is: "corms are ovoid, depressed-globose with fibrous tunic finely reticulated. Cataphylls are membranous. Leaves, subhysteranthous or synanthous but if absent at anthesis then developing after the flowering, are green or slightly grayish and glabrous. Flowers are mid-lilac to purple with darker veining, the external pale silvery or buff coloured throat lilac, glabrous. Perigonium tube, white or lilac; segments sub-equal, oblanceolate, obtuse, the inner usually slightly smaller than the outer. Filaments, glabrous; anthers yellow. Style divided into 3 red thickened branches, and about equaling the tips of the anthers, arising at a point at or just above the throat of the flower, below the base of the anthers. Capsule oblong, on a short pedicel just above ground level; seeds reddish-purple, sub globose" (**Table 1**).

Crocus pallasii Gold. (1817). It is rather a variable species with pale lilac flowers and rather short, inconspicuous style branches, less than half as long as the perigonium segments. It occurs from the Balkans to Iran and from the Crimea to S. Jordan. Over this large area, it varies considerably, four subspecies being recognized.

Crocus asumaniae B. Mathew *et al.* (1979). Corms are ovoid with tunic fibrous extending at the apex of the corm into a neck. Cataphylls are membranous. Leaves are hysteranthous or with the tips just showing at anthesis, slightly greyish-green, glabrous. Flowers are white, occasionally with dark veins near the base of the segments, rarely very pale lilac; throat whitish or pale yellow, glabrous. Perigonium tube white; segments sub-equal, oblanceolate or narrowly elliptic, obtuse to acute, the inner slightly smaller than the outer. Filaments white or pale yellow, glabrous; anthers yellow. Style divided into reddish-orange clavate branches, each considerably exceeding the anthers and at least half the length of the perigonium segments, arising at a point well above the base of the anthers. Capsules are ellipsoid; seed reddish-purple, subglobose.

Crocus mathewii Kerndorff and Pasche (1994). Corm 16-mm in diameter depressed globose, with tunics fibrous extended into a neck. Cataphylls are silver-white, membranous. Leaves are hysteranthous dark green, sparsely ciliate. Fragrant flowers are white or rarely pale lilac-blue, often stained deep violet at the base of the segments inside and outside; throat violet, pubescent. Prophyll, bract and bracteole present. Perigonium tube, usually violet in the upper part, paler to almost white lower down; segments sub-equal, ovate to obovate, obtuse to slightly acuminate, the inner slightly smaller than the outer. Filaments, white, glabrous; anthers, usually clearly exceeding but sometimes equaling or rarely shorter than the anthers and less than half as long as the length of the perigonium segments, arising at a point well above the base of the anthers. Ellipsoid capsule raised on a short pedicel above ground level at maturity; seeds purplish-brown, globose.

C. naqabensis Al-Eisawi and Kisawi (2001). Species nova for Jordan Flora (Al-Eisawi 1985, 2001) has features similar to *C. pallasii* but its corms have reduced tunics that do not form a neck. Moreover, flowers have a globous throat. *C. naqabensis* is also closely related to the endemic *C. moabiticus* and *C. cartwrightianus* from which it differs for the style branches which are not more than half as long as the perianth segments, for the absence of tunic necks and the glabrous throat.

According to the above reported systematic criteria *C. sativus* belongs to Family Iridaceae, genus *Crocus*, subgenus *Crocus*, section A, Series *Crocus*, Series type: *Crocus sativus***.** Section of *C. cartwrightianus*. The comparison of the autumnal flowering *Crocus* spp. indicates a strong similarity of *C. sativus* to *C. thomasii* and *C. cartwrightianus* (**Table 1**). Their morphology and dimensions are smaller than in triploid *C. sativus.* However comparison of the species on the basis of morphological characters does not allow a precise distinction of the taxa. In polyploids in fact each member of a gene pair do not contributes equally to expression level in the new phenotype (Osborn *et al.* 2003; Otto 2003). And the analysis of flower pigment composition of *Crocus* spp. and cultivars used as chemotaxonomy (Nørbaek *et al*. 2002) generally supports the classifications of Mathew.

CYTOLOGY AND CYTOGENETICS

The first cytological studies aimed at investigating relationships among *C. sativus* and related wild species date back to 1931 (**Table 2**). In a list of chromosome number Sugiura (1931) reported *Crocus sativus* with 2n=24 chromosomes; the same number of 24 somatic chromosomes was detected by Morinaga and Fukushima (1931), in the root-tip cells of *C. sativus.* Subsequently, Mather (1932) found in saffron 2n=15 and 2n=14. Karasawa (1933, 1940) for chromosome number of *Crocus* including *C. sativus* and related species, reported 2n=24 for *C. sativus* which proved to be autotriploid 2n=3x, x=8. Pathak (1940), Feinbrun (1958), Brighton (1977), Mathew (1977), Chichiriccò (1984), Ghaffari (1986), Ebrahimzadeh *et al.* (1998) carried out numerous studies on chromosome number and karyotype of *C. sativus* and on the allied species chromosome behaviour at meiosis as well as on chromosome morphology and composition. The results of these studies confirmed *C. sativus* as triploid with 2n=3x=24, x=8. A similar basic genome x=8 but with 2n=16 has been found in *C. cartwrightianus*, *C. thomasii*, *C. hadriaticus* (**Table 3**). The karyotype construction on the basis of chromosome morphology and their DNA content made it possible to interpret the genome structure as that of an autotriploid (Chichiriccò 1984). However, more recent contributions on the chromosome structure of *C. sativus* accessions from different cultivation areas combined with the use of markers and fluorochromes to prove the chromosome base composition concluded with the hypothesis that saffron may be an allopolyploid (Agayev 2002; Nørbaek *et al.* 2002; Fernández 2004). In addition, Agayev *et al.* (2010) comparing karyograms structure evidenced differences between *C. sativus* L. "Kashmirianus" 2n=24 and usual *C. sativus* 2n=24, assuming that the first is a cultivar genetically not identical to the latter.

All these studies were important in discriminating the diploid *Crocus* species autumnal flowering from the diploid spring flowering. To autumnal flowering *Crocus* are diploid species with genome 2n=16, x=8, as *C. cartwrightianus*, *C. thomasii*, *C. hadriaticus*, *C. oreocreticus*, *C. moabiticus*, *C.*

Table 2 Chromosome number in *Crocus sativus* L.

Chromosome	References
2n = 24	Morinaga and Fukushima 1931
2n = 24	Sugiura 1931
2n = 14, 15	Mather 1932
2n = 3x = 24	Karasawa 1940
2n = 3x = 24	Pathak 1940
2n = 16, 20, 24, 40	Karasawa 1943
2n = 20, 22, 28	Pogliani and del Grosso 1971
2n = 3x = 24; x = 8	Mathew 1977, 1999
2n = 24	Estilai 1978
2n = 3x = 24; x = 8	Chichiriccò 1984
2n = 3x = 24; x = 8	Ghaffari 1986
2n = 24	Dhar *et al.* 1988
2n = 24	Khan 1996
2n = 16, 24	Ebrahimzadeh *et al.* 1988
2n = 3x = 24; x = 8	Agayev 2002

Table 3 Cytology of the species in *Crocus sativus* series.

***Crocus* species**	**Author's name**	**year**	**Chromosome number**	**Reference**
C. asumaniae	B. Mathew & T. Baytop	1979	2n = 26	Mathew 1999
C. cartrwrightianus	W. Herbert	1843	2n = 16	Brighton 1977
C. hadriaticus	W. Herbert	1845	2n = 16	Brighton *et al*. 1973
C. mathewii	H. Kerndorff & E. Pasche	1994	2n = 16	Mathew 1999
C. moabiticus	F. Bornmuller & J.E. Dinsmore	1912	2n = 14	Kerndorff 1988
C. naqabensis	D. Al-Eisawi	2001	2n = 14	Al-Eisawi 2001
C. oreocreticus	B. L. Burtt	1949	2n = 16	Brighton *et al.* 1973
C. pallasii subsp. *pallasii*	K. L. Goldbach	1817	2n = 14	Mathew 1999
dispataceus	E.A. Bowles	1982	2n = 14	Mathew 1999
haussknechtii	(E. Boissier & Reut. x Maw) B. Mathew	1977	2n = 16	Jacobsen and Ørgaard 2004
turcicus	B. Mathew	1977	2n = 12	Mathew 1999
C. sativus	C. Linnaeus	1753	2n = 3x= 24	Agayev 2002
C. thomasii	M. Tenore	1826	2n = 16	Brighton *et al.* 1973

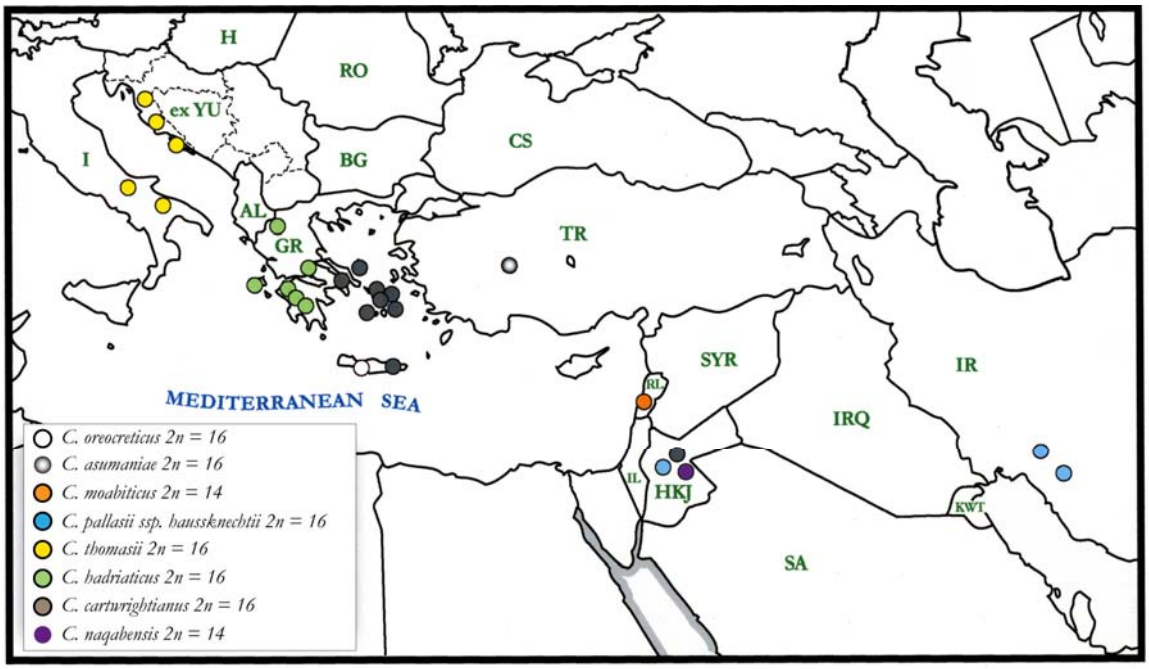

Fig. 3 Geographical distribution of diploid autumnal flowering *Crocus* species to *C. sativus* allied.

pallasii var. *haussknechtii*, *C. mathewii*, others as *C. pallasii* with 2n= 12, 14, 16, *C. naqabensis* with 2n=14, *C. asumaniae* with 2n=26. Comparison of the karyotypes of the allied *C. sativus* species demonstrated that *C. cartwrightianus* is one progenitor only in the case of autotriploid (Mathew 1982, 1999; Grilli Caiola *et al*. 2004; Zubor *et al*. 2004; Frizzi *et al*. 2007), or *C. thomasii* (Chichiriccò 1984) or more than one species. In the hypothesis of allopolyploidy e.g. *C. cartwrightianus*, *C. hadriaticus*, *C. oreocreticus* (Jacobsen and Orgaard 2004; Agayev *et al*. 2010); or *C. thomasii* and *C. pallasii* or *C. cartwrightianus* and *C. pallasii* (Tammaro 1990) are the candidates as progenitor by cross processes.

GEOGRAPHIC DISTRIBUTION

Further insights into saffron's origin arise by comparing the geographic distribution of the allied species of *C. sativus*. As shown in **Fig. 3** many diploid species of *Crocus* occur in the Mediterranea area (Baytop *et al.* 1975; Burtt 1948; Feinbrun and Shimida 1977; Jacobsen and Orgaard 2004; Kerndorff 1988; Mathew and Baytop 1976; Mathew 1982, 1999). Among them are *C. thomasii* Ten., *C. cartwrightianus* Herb., *C. hadriaticus* Herb., *C. oreocreticus* B. L. Burtt, *C. pallasii* Gold, *C. naqabensis* Al-Eisawi. *C. cartwrightianus* is distributed in south eastern Greece, *C. hadriaticus* is western Greece; *C. oreocreticus* is endemic to Crete in limestone formations up to 200 m high; *C. thomasii* occurs in Italy and in mountains of the Adriatic coast; *C. pallasii* has a wide distribution in South Turkey and Nord Syria, as do the four subspecies; *C. asumaniae*, Turkey. Included among the Mediterranean *Crocus* is also *C. mathewei*, recently identified and distributed in a small region in southwest Turkey.

Greece and Turkey (Baytop *et al*. 1975; Mathew and Baytop 1976) are the richest countries with the highest number of *Crocus* taxa. Among the 80 species listed in Mathew (1982) 40% of total *Crocus* diversity is in Greece (Tsokdouridis *et al*. 2009; Valamoti and Sarpaki 2009). The other centre rich in *Crocus* is Turkey whose flora comprises 32 *Crocus* species; 18 of them are endemic while 30 are species cultivated as ornamental plants (Arslan *et al*. 2007).

Combining the historical records with systematic, cytogenetic and geographic distribution data (species with 2n=16, x=8) it is possible to identify the saffron cited by ancient historians as most probably being *C. cartwrightianus* or *C. thomasii* or *C. hadriaticus*.

BIOCHEMICAL AND MOLECULAR DNA ANALYSIS

The systematics of *Crocus* genus based on morphological, geographical, cytological data does not allow a precise separation of some taxa and phylogenetic relationships. This is complicated by hybridization and mutation processes resulting from different karyotypes obtained in *C. sativus* and other species from different countries. Since 2000, molecular studies have been undertaken both on *C. sativus* aggregate and on phylogeny of *Crocus* genus (Frello

et al. 2004). The above division has undergone revision, by means of DNA analysis using the cytofluorimetric method (Brandizzi and Grilli 1998), and by sequence data from five plastid regions (Petérsen *et al*. 2008), Amplified Fragment Length Polymorphism (AFLP) (Zubor *et al*. 2004), and Random Amplification of Polymorphic DNA (RAPD) (Grilli *et al.* 2004).

Cytofluorimetric analyses of nuclear DNA of different *C. sativus* accessions, from Italy, Spain, Israel, and Holland have revealed some morphological differences but no differences in DNA content and base composition (Brandizzi and Grilli Caiola 1998). In addition triploid content and DNA quality of *C. sativus* was compared to that of diploid *C. cartwrightianus*, *C. thomasii*, *C. hadriaticus*. The analyses performed by flow cytometry and by zymograms of SOD and peroxidases have indicated that there is a scarse intra-specific variability among the analysed species and that *C. sativus* could originate from cross-phenomena in *C. cartwrightianus* with another fertile species. Nuclear DNA analyses by RAPD technique on leaves of the above-listed *C. sativus* accessions and diploid *Crocu*s species in plants grown in the same site and utilizing 21 (10-mer) primers did not identify any genomic redundant differences. No differences in corms of saffron from l'Aquila were detected. DNA polymorphism-based taxonomy with the use of AFLP method has provided further results to confirm that the closest relative among the allied of *C. sativus* is *C. cartwrightianus*. However *C. thomasii* also shows similarity to *C. sativus* and *C. cartwrightianus.* Thus, the AFLP method proves that out of six species from series *Crocus*, it was *C. cartwrightianus* and *C. thomasii* which showed an over 70% similarity to *C. sativus* and to each other. This value suggests that among the members of the *Crocus* genus, the closest relationship is between these three species.

Studies by RAPD and microsatellite analysis of DNA in forty three isolates of *C. sativus* from 11 different countries have confirmed that *C. sativus* accessions result identical clones at molecular level (Rubio-Moraga *et al*. 2009). The tandemly repeated DNA sequence family (Frello *et al.* 2004), the internal transcribed nuclear ribosomal regions (ITS1 and ITS2), internal trnH and psbA genes of cpDNA, as well as the 5.8S and 16S ribosomal genes (Tsoktouridis *et al.* 2009) were all used to investigate the phylogeny of the genus *Crocus*. Frello *et al.* (2004) have based their phylogenetic studies on sequences from five plastid regions. They examined 86 of 88 recognized species of *Crocus* and the analysis of a total of 222 phylogenetically informative characters. A clonal origin has recently evidenced by Fluch *et al.* (2010) showing a same alleles by Iranian and Spanish whereas samples from Germany, Austria, Italy and France shared an other allelic combination. Most of these studies have been carried out to establish the possible genetic variations among different *C. sativus* accessions as well as to establish both the phylogenetic relationships of *C. sativus* to other *Crocus* species of the same group, the autumnal flowering diploid with a base genotype x= 8, similar to that of *C. sativus*.

Results of the DNA studies have revealed small or no differences between *C. sativus* DNA and that of the allied species *C. cartwrightianus*, *C. thomasii* and *C. hadriaticus.* However *C. cartwrightianus* is considered the most probable parent of the triploid *C. sativus* (Frello *et al.* 2004). Similar results have been obtained by Frizzi *et al*. (2007) on analyzing the allozyme differentiation in *Crocus* species genome. Phylogenetically *C. sativus* appears closer to *C. cartwrightianus*, although definitive conclusions are lacking.

REPRODUCTIVE BIOLOGY

Vegetative multiplication

Many studies have been dedicated to the biology of saffron reproduction. Due to its triploidy saffron is usually multiplied by means of selected corms. This process is carried out by man. Only rarely have seeds in *Crocus sativus* been reported in nature (Piccioli 1932; Di Crecchio 1960), despite the abundant production of sexual reproductive structures such as pollen and ovules.

Saffron has a life cycle characterized by a long summer break and vegetative activity from autumn to spring. The plant, after the loss of leaves, survives the summer as an underground corm. During this period the corm prepares the buds that will originate the new leaves and flowers. The leaves sprout from apical buds on a short stem and are embedded by whitish bracts. From other apical buds originate the flowers, which are frequently hysterantic or appear just after the leaves do. At the base of mother corms smaller new corms also occur, creating a new plant which normally produces only leaves. The number of new leaves, flowers and daughter corms depend on the dimensions and age of the mother corms, on cultivation methods, and on environmental conditions. The number and quality of buds originating leaves, flowers or young corms depends on the amount of resources that the mother corm is able to accumulate during the vegetative period. Recent research (Agayev *et al.* 2009) considers the saffron as a bulk of clones accumulated during the millennial cultivation of the plant by man. The selection of the bigger corms is proposed as a system for improving cultivated saffron (Agayev *et al.* 2009). Although the selection of the bigger corms is a common practice carried out by saffron farmers over many countries, a clonal selection of corms with high yield capacity is a programme with great potential. In fact, the corm after some years of cultivation in the same field does not produce flowers (Tammaro 1987, 1990). The number of flowers depends on various factors including the age of the corms. Such considerations date back to the law according which, in a plant, the allocation of resources between vegetative and reproductive phase are correlated. If the plant uses more resources in producing flowers, in the following years it will have fewer resources for vegetative production such as corm, stem, leaves, flowers, and fruits. More recently Schnittler *et al*. (2009) described the bulbils versus seed production in the liliacean *Gagea*. These observations shed light on the very difficult conditions that saffron has to overcome in surviving as a wild plant in the natural state. Observations on corms left in field for 10 years without cultivation practices (Grilli Caiola 2005) demonstrated that the corms decreased their dimensions and flower production over the years. The smaller corms had only one leaf, whilst larger corms had 2-8 leaves. Thus, after ten years without cultural intervention, saffron corm loses its vigor and degenerates (Grilli Caiola 2005), giving rise to smaller corms unable to flower and produce new corms. As far back as Herbert (1847) hypothesized that wild saffron disappeared as a consequence of changes occurring in its natural habitat.

Sexual reproduction

The triploid condition of saffron causes an anomalous pairing of the chromosomes at the prophase of meiosis, and an irregular distribution of chromosomes at methaphase with a consequent infertile gametes production (Chichiriccò 1987). However, often in the triploids, pollen and ovules do not exhibit the same behaviour. Generally infertility in uneven polyploids is much higher in the pollen when compared to the ovules. This aspect has been studied in saffron and compared to the behaviour of the allied diploid species by focusing on the structural organization of the reproductive structure as well as on the process of compatibility and incompatibility among species of the *Crocus* group.

a) Microsporogenesis and pollen. Studies on pollen have demonstrated the high number of anomalous pollen grains of *C. sativus* in terms of both grain dimensions and shape. During meiotic division in saffron many abnormalities occur which are also commonly found in other triploid species (Chichiriccò 1984). Microspores often display cytoplasmic degeneration or cellular deformation; consequently they do not complete meiosis or produce anomalous micro-

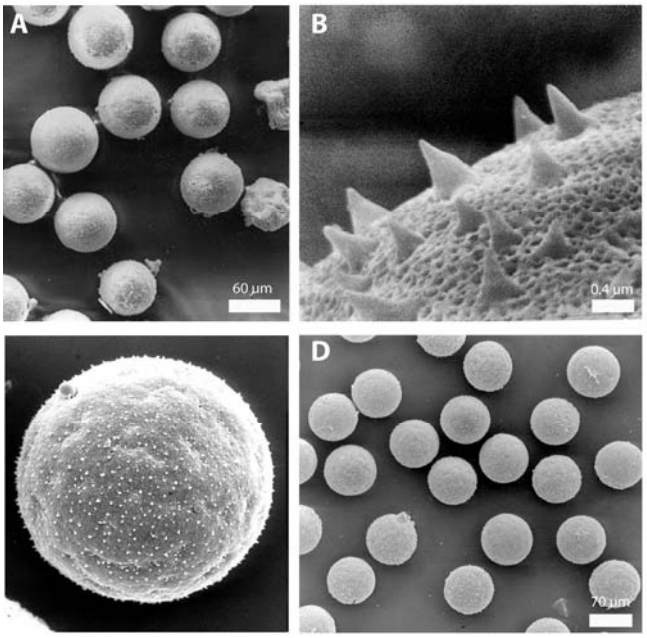

Fig. 4 Pollen grains at SEM (**A**) *C. sativus*; (**B**) spinulae on pollen of *C. sativus*; (**C**) *C. cartwrightianus*; (**D**) *C. thomasii*.

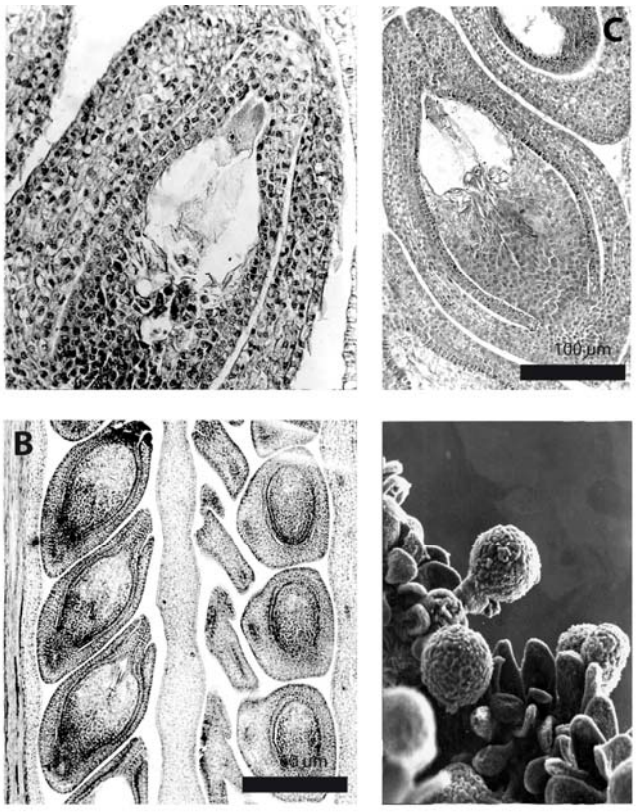

Fig. 5 Ovary and ovules at LM (**A**) Ovule in longitudinal section of *C. sativus*; (**B**) Ovary with ovules and (**D**) a single ovule in *C. cartwrightianus*; (**C**) stigma of *C. sativus* with autopollen.

spores. Afterwards many spores are aborted or the pollen grains are of different size and also often malformed (Chichiriccò 1987). Observed at SEM (Grilli Caiola *et al*. 1985; Grilli Caiola 1999; Grilli Caiola *et al*. 2001; Grilli Caiola 2004), saffron pollen is an elliptical shape with variable size (65-140 µm) and lacking germinative pores or furrows (**Fig. 4A**). About 40% of pollen grains are anomalously shaped, collapsed or broken. The exine surface shows many randomly-disposed spinulae (**Fig. 4B**) and lipid droplets as well numerous perforations of varying size and form. Microsporogenesis and pollen organization in the diploid allied species are generally regular and shape and structure of pollen is similar. Inaperturate pollen is also common in other *Crocus* species (Grilli Caiola *et al*. 1993; Grilli Caiola 1994, 1995).

By comparing generative cell structure, viability and germination *in vitro* and on stigma (**Table 1**), it turns out that saffron has a higher percentage of anomalous pollen grains as well as a lower percentage of viable and germinating ones, both *in vitro* as well *in vivo*, when compared to the diploid species *C. cartwrightianus* (**Fig. 4C**), *C. thomasii* (**Fig. 4D**) and *C. hadriaticus.* The percentage of germination pollen in vitro of *C. sativus* proves to be 20% (Grilli Caiola 2005) higher as opposed to the 10% reported in Karasawa (1933). Only 0.4% of *in vitro* germinating pollen grains showed regular pollen tubes, most of the others being accompanied by numerous morphological anomalies such as forked tube, sister-shaped tube, swelling at their base and apex, spiraled pollen tubes and thinning at their ends (Grilli Caiola and Chichiriccò 1982). Significant differences were observed in the viability and germination of pollen collected from flowers at different developmental stages such as in cataphyllic flowers, young flowers and open flowers (Grilli Caiola *et al*. 2001). The highest germination percentage turned out to be from pollen taken from anthers of mature flowers, at the anthesis.

b) Megasporogenesis and embryo sac. At anthesis gynoecium of the above considered *Crocus* species have stigma of dry type, with papillae covered by a thick continuous cuticle. Stigmas are erect until anthesis but as the flower opens they bend downwards. The style is internally made up of three separate channels, forming a single cavity which in the main tract is lined with a layer of secretory cells extending to the ovary where the stylar cavity opens into three locules, being the ovary tricarpellar and trilocular with introvarian nectaries. Along the axial region of the locules are placentae on which the ovules are obliquely attached, two rows of 9 ovules for each locule. Ovules are anatropous and bitegmic with a large hypostase. The external integument extends beyond the internal one and forms a narrow micropilar canal. Megasporogenesis occurs during early upper sprouting and an embryo sac appears in the ovules of young flowers still enveloped by cataphylls, so there are no differences between embryo sacs of young and open flowers (Grilli Caiola *et al.* 2001). In *C. sativus* during meiotic division an irregular assortment of chromosomes was observed and the resulted megaspores were numerically variable (4-6) and genetically unbalanced. About 90% of ovules developed an embryo sac which is broad and 7-nucleate (*Polygonium* type) (**Fig. 5A**). Frequently the ovules do not reach a fertilizable stage due to unsuccessful megasporogenesis or megaspore development. About 12% of ovules develop a small embryo sac with variable number of cellular nuclei, often no more than four; around 18% of ovules are lacking an embryo sac, and show proliferation of nucellar tissue which increases up to the micropyle. Degeneration of the embryo sac is not frequent and in this case the embryo sac contains abundant granular material and sometimes micronuclei also (Chichiriccò 1987). Megasporogenesis in *C. sativus* is similar to that reported in other *Crocus* species (Rudall *et al*. 1984). The embryo sac is 7-nucleate when mature and contains a substance which stains red with Poinceau especially during the initial developmental stages. The ovary of *C. cartwrightianus* contains two rows of 9-10 ovules for each locule and the inner integument is made up of 4-5 layers (**Figs. 5B, 5C**); *C. thomasii* ovary contains two rows of 12-14 ovules for each locule and each ovule has an integument 4-5 cell layers thick at the micropyle; *C. hadriaticus* ovary has 6-9 ovules for each locule (Grilli Caiola and Chichiriccò 1991; Grilli Caiola *et al*. 2001). In all the three diploid species the megasporogenesis and embryo sac development is regular with a normal development of a female gametohyte including an egg cell, synergids, a central cell, and an antipodal apparatus (Chi-

chiriccò 1989a, 1999; Grilli Caiola *et al.* 2001).

Our recent SEM observations of the ovule surface of *C. sativus*, *C. cartwrightianus* and *C. thomasii* (unpublished data) have shown that the ovule has smooth epidermal. In *C. sativus* ovule has dimensions similar to *C. cartwrightianus* but longer and larger than in *C. thomasi*. In addition in these two species the ovule surface is formed of larger cells, swollen or sunken. In *C. thomasii* (Grilli Caiola, pers. comm.) epidermal cells are smaller, with parallel transversal walls, and a rather flat surface.

Pollination

At anthesis the saffron flower shows adhering tepals, closed anthers and initially erect red stigmas. When the flower opens the stigmas downland and the anthers dehisce. During anthesis the tube of perigonium up to the throat is filled with a liquid nectar originating from intraovarian secretory canals. The secretion rises to the upper part of the ovary and accumulates inside the perigonium tube during the night. During the morning, at increasing temperature, the tepals separate and expose the style with the stigmas downlanding. Our observations from 2000 to 2006 confirmed that insects visiting the flowers of *C. sativus* can be *Bombus sylvestris* and *Apis mellifera.* These pronubes seem to work in various ways according to the environment. Initially we studied the activity of *Bombus* in a small area where *Crocus sativus* and *C. cartwrightianus*, *C. hadriaticus*, *C. oreocreticus* were grown in soil and *C. thomasii* in pots. Usually a *Bombus* group of ten or more individuals appeared at noon of sunny days when the temperature had risen, so facilitating the opening of the flowers and the anthers and the emission of an intense perfume. In these conditions *Bombus* flew from flower-to-flower collecting pollen. On visiting flowers of different *Crocus*, *Bombus* collected mainly pollen, not nectar. From this point of view it seems a good pollinator. *Apis* have been observed on balconies of a private home in the city-center where a series of pots with saffron and allied *Crocus* species were cultivated. Bees arrived in the early morning when flowers were still closed. They were able to separate the adhering tepals and get down into the style to collect the secreted liquid. The bee's main interest was in collecting nectar. They are quick and very active on visiting numerous flowers, when the anthers are still closed. We followed the activity of these insects for many days, ascertaining that bees are able to overcome heavy difficulties represented by flower-covers applied to prevent free pollination. No fruit was obtained from saffron plants visited by bees. These observations seem to indicate that bees are not as good pollinators for *Crocus* as *Bombus* are. It is impossible to establish if these observations could indicate a different territory occupation by different pronubes or other mechanisms as those indicated by Dudareva *et al*. (2006). It is noteworthy that the same insects prefer saffron and *C. cartwrightianus* to other allied species. The absence of pollen transport from bees is revealed by the absence of pollen in honeys from regions where saffron grows such as Aquila in Italy. In Italy only a few *Crocus* pollen have been found in the honey from some Alpine, North and Central regions (Ferrazzi 1991). This contradicts what Columella reports in *Res Rustica* according to which "*The cultivation of Crocus from Corycius and Sicily is useful in dying and perfuming the honey*". In open fields it may be possible that bees or bumble-bees carry out pollination in saffron with consequent formation of capsules and seeds as reported by Piccioli (1932) in saffron cultivations of L'Aquila. According to our observations free fertilizations could have occurred in saffron grown near *C. cartwrightianus*, as well in *C. cartwrightianus* and *C. thomasii* when visited by *Bombus*.

Compatibility and incompatibility

Spermatophyta have evolved a genetic system of self-incompatibility (SI) in order to prevent inbreeding so favouring out-crossing. However out-crossing is limited by incompatibility between different species. The mechanisms of intra- and inter-specific breeding are regulated in different ways in different plants. Sometimes structural mechanisms regulate autogamy as in plants with different floral morphs. In other cases self-incompatibility is based on signals at the level of the pro- or postgamic phase. The responsibilities for the inhibition of pollen germination or pollen growth inside the stigma or style vary according to the group of angiosperms. In some cases the cause is the Ca^{2+} ions, in others RNAse, and in still others compounds secreted by cells of the stylar canal. Inside *Crocus* genus SI systems have been investigated in species of the *C. sativus* group, such as *C. sativus*, *C. thomasii*, *C. cartwrightianus*, *C. hadriaticus*, *C. oreocreticus*, the goal being to ascertain the mechanism of self-incompatibility (Chichiriccò 1989b, 1996) and establish the relationships within the different species of the group. Overall the results of this research are important for verifying the possibility of establishing the genetic compatibility of *C. sativus* with one or more diploid species. In addition the analysis of seeds obtained by means of crossing saffron with pollen of diploid allied makes it possible to compare the possible similarities to or differences from the maternal plant (Grilli Caiola *et al*. 2010). Results of these experiments have indicated that *C. sativus* is self-and allosterile (**Fig. 5D**); *C. thomasii* is self-incompatible but in outcrossing produces 86% fertilization and 84.5% seed set; *C. cartwrightianus* is self-sterile but out-fertile with a high percentage of fertilization; *C. hadriaticus* shows the highest percentage of ovules with embryo sac and 87% fertilisation after outcrossing, but 0% fertilization after self-pollination. *C. oreocreticus* has a low percentage of ovules lacking embryo sac and has 70% fertilization in out-cross and 40% in self-cross, the highest value observed in the tested species. As regards the interspecific crossing it turns out that pollen of *C. sativus* does not germinate or grow in any of the tested stigmas, but is fertilized by pollen of *C. thomasii* (16.8%), *C. hadriaticus* (8.5%), *C. oreocreticus* (11.0%), *C. cartwrightianus* (10.2%). *C. sativus* seed sets after crossing pollination with *C. thomasii* or *C. cartwrightianus*. The seeds of *C. sativus* x *C. cartwrightianus* were grown for 4 years and their germination and development was studied and compared to seeds obtained from *C. cartwrightianus* outpollinated by hand (Grilli Caiola 2005). Regarding germination, seedling, and young corms formation, the behaviour of the seeds of both the species is very similar.

On the whole the results of the numerous experiments performed on the species of *C. sativus* group and on species of other *Crocus* group indicate that the ovarian self-incompatibility (SI) is widespread within the genus *Crocus*, this resulting in a partial or complete suppression of self-fertilization. Moreover the post-zygotic SI mechanism as well as post-zygotic mechanisms of unknown nature seem to be recurrent and both are responsible for seed abortion. The interspecific ovarian incompatibility concerns only unrelated crosses. Crosses between related fertile species succeed both in fertilization and seed-set (Grilli Caiola and Zanier 2005).

Regarding the mechanisms of incompatibility, studies on RNase activity indicate that in *Crocus* RNases are not responsible for mechanisms of rejection of incompatible pollen tubes. Similar results have been obtained in studies on stylar peroxidase activity against the incompatible pollen tubes (Zanier and Grilli Caiola 2001). The Ca^{2+} also seems not to be responsible for stylar incompatibility (Brandizzi and Grilli Caiola 1996). Investigations using various methods seem to confirm that the cause of arrest of pollen tubes in the examined *Crocus* species is localized at the level of lower part of stylar and upper part of the ovary. Concerning the composition of this mechanism, it seems most probable that it is a glycosilate compound.

Pollen grains of *C. sativus* germinate in a low percentage on the stigma, and pollen tubes do not reach the ovary. These results could be proved for the triploid condition of this species which would negatively condition the correct

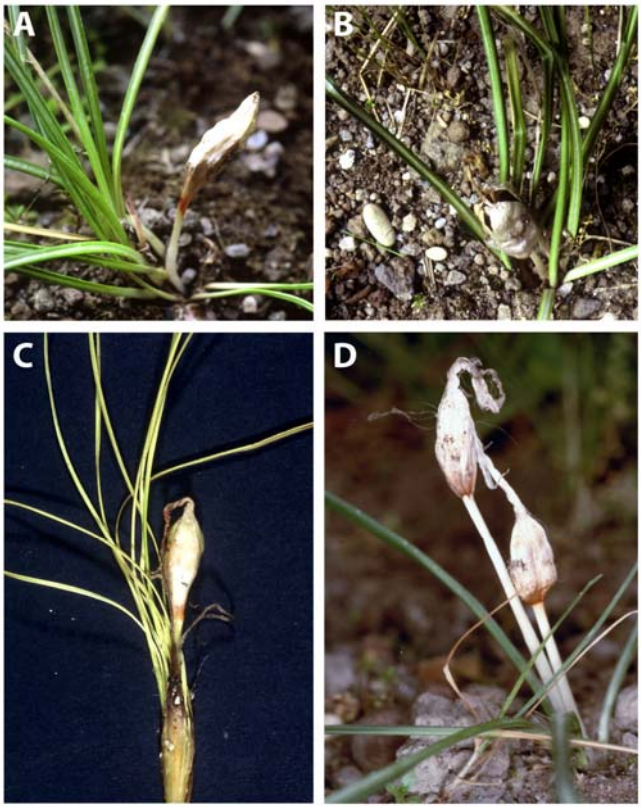

Fig. 6 Capsules (**A**) *C. sativus* x *C. cartwrightianus*; (**B**) *C. cartwrightianus*; (**C**) *C. thomasii*; (**D**) *C. hadriaticus*.

pollen germination and pollen tube development. *C. cartwrightianus* proves to be mostly an out cross-fertile. This is because, firstly, the pollen germinates on the stigma in a higher percentage after out cross-pollination in respect to self-pollination. Secondly the self-pollen tubes are blocked in the basal part of the style. Finally, the capsules are produced only by out cross-pollinated flowers.

Saffron, although sterile, produces a very high amount of pollen and floral structures which are ineffective for the purposes of seed production. This is unintelligible if we consider the high amount of energy allocated to produce floral organs and function (Cruden 2000). Production of bulbils versus seeds has been detected in *Gagea* (Liliaceae) (Schnittler *et al*. 2009).

Fruit set, seed set and seed coat microstructure

The successful pollination in *Crocus* produces a capsule maturing at soil level. *Crocus sativus* hand-pollinated with pollen of *C. cartwrightianus* originated a large capsule maturing in May and visible above ground. The capsule is erect and provided with a peduncle which connects the fruit to the basal underground corm (**Fig. 6A**). When mature the capsule opens by means of apical breakings and the seeds are dispersed on the soil after the capsule has dried. Similar behaviour is detected in the capsules of the diploid *C. cartwrightianus* (**Fig. 6B**), *C. thomasii* (**Fig. 6C**), *C. hadriaticus* (**Fig. 6D**). Seed surface microstructure has been used in many angiosperms to discriminate critical taxa (Barthlott 1981; Karcz *et al.* 2000; Zeng *et al.* 2004). Seeds from *C. sativus* pollinated with *C. cartwrightianus* were analysed under a light and scanning electronmicroscope in order to establish the similarities to or differences from the seeds of diploid *C. thomasii*, *C. cartwrightianus*, *C. hadriaticus* grown in a similar site and hand out-pollinated. The seed surface morphology, the inner structure and the dimensions of the compared species are all reported in Grilli Caiola *et al*. (2010). The seeds differ in dimension, colour, and raphe development. However, the basic morphology and structure organization are similar in all the considered species. Seed

Fig. 7 *C. sativus* x *C. cartwrightianus*: (**A**) 1) small roundish corm from germinated seed; 2) Long corms from seed after three years grown. (**B**) *C. cartwrightianus*: 1) small roundish corm from germinated seed; 2) Long corm form from seed after three years grown; 3) Cormlets formed at the basis of the corm in 2 B).

derives from an anatrope ovule which is curved and the micropyle occurs near the funicle (Grilli Caiola and Chichiriccò 1991; Grilli Caiola *et al*. 2001). Due to this position, the seed carries a linear lateral wing-like protrusion known as raphe that appears as a double sheet open along the side length revealing a small opening. The raphe departs from the basal funicle and extends longitudinally along a side of the seed until the apex. The ovule is bitegmic and its inner integument protrudes as a multilayered structure. At this point in the mature seed, a caruncle is more o less evident in different *Crocus* species. The outer tegument proves to consist of seven to ten cell layers. The seed epidermis is covered by a mass of hairs of cylindrical shape, erect or curved or elongated. Endosperm has thin epidermal layer with small cells and the parenchyma with large cells radially elongated, with walls thickened by pectic, giving the seed a hard structure. Storage reserves consist of lipid drops, rare starch granules and abundant proteic granules. Seeds of *C. sativus* have the higher dimensions respect to the diploid allied species. This is in according with the triploid condition of the saffron. The seed coat surface has hairs which are similar in *C. sativus* and *C. cartwrightianus* but different from those of *C. thomasii* and *C. hadriaticus* (Grilli Caiola *et al.* 2010). The seeds of *C. sativus* obtained as reported above were germinated and grown in pots for four years. The same treatment was used to germinate and grow the seeds of *C. cartwrightianus* and *C. thomasii* (Grilli Caiola 2005). Results of the observations indicated that saffron seeds were viable and germinated in as high percentage as those of diploid species. In addition, the morphology and growth behaviour of the germling and seedling were similar in *C. sativus* and *C. cartwrightianus*. Seedlings of the first year had a short white prophyl from which a green leaf emerged. During growth a small roundish corm, without tunic, formed at the base of the green leaf. This corm after a dormant period produced a new green leaf and at the end of growth formed a larger new corm with reticulate-fibrous tunic. After three years of growth the corm formed three new cormlets at the base. The corms from the second year were small, ovoid in shape, and protected by reticulate tunics (**Figs. 7A, 7B**).

CHROMOPLAST STRUCTURE AND PIGMENT COMPOSITION

The commercial product and use of saffron comes from the dried stigmas, appreciated for a special colour, taste and aroma (Carmona Delgado *et al.* 2006). Saffron spice gives dishes and other material the yellow colour, bitter taste and intense aroma. Although the main use of saffron is culinary, also important are its pharmacological properties and role in medicine (Abdullaev 2003). The characteristic of colouring, flavouring, and aromatising are related to the presence in the red stigmas of glycosyl compounds, esters of crocetin carotenoids, picrocrocin, and an extensive group of cetons and terpenic aldehydes, safranal being the most outstanding.

Saffrons colour is due mainly to carotenoids, molecules generally insoluble in water but capable of generating an intense colour when in hot water. Carotenoids form a group of pigments very common in plants. They are formed of a long aciclic chain with double conjugate bonds such as lycopene, or, more frequently, of a chain terminated by six or five rings on one or both ends. Due to numerous double bonds carotenoids present various geometric isomers. They can exist as apocarotenoids in a short chain after elimination of some terminal rings and reduction in size of the molecule. In addition acid molecules of crocetin can be exterified by glucose, gentibiose and neapolitanose, giving rise to numerous esters of crocetin present in saffron. According to a hypothesis (Bouvier *et al.* 2003) crocetin in saffron derives from zeaxanthin precursor which breaks at both ends to generate crocetinaldehyde which is oxidized to give rise to crocetin. Subsequently the glycosylation occurs through the action of a glycosyltransferase.

Saffron's characteristic bitter flavour is due to the presence of picrocrocin, a flavonoid compound. It originates through oxidative degradation of zeaxanthin and is in turn the precursor of safranal, the main compound of the saffron aroma. Picrocrocin is present in saffron in high amounts, about 13% of the dry weight.

Saffron's aroma was for a long time attributed to safranal generated from picrocrocin, but recently many other minor compounds are considered responsible for the saffron aroma. The aroma is the most appreciated component of the saffron spice, although the methods to reveal the presence of such compounds are still ongoing.

The above reported compounds are sited in organelles, the chromoplasts, occurring in the epidermal and parenchyma cells of stigma and the coloured upper part of the style. The structure of chromoplast have been examined in *C. sativus* (**Fig. 8A**) (Grilli Caiola and Caprilli 1983; Grilli Caiola 2004; Grilli Caiola and Canini 2004) as well as in the related species *C. cartwrightianus* and *C. thomasii* (Grilli Caiola and Canini 2005). In saffron the chromoplasts occur only in the red-coloured parts of stigmas and style. They appear in the red stigma of very young floral buds, and originate from amyloplasts, the only plastids present in the colourless basal portion of the style as well as in the parenchyma of ovary and resting corm. Transitional forms of amylo-chromoplasts occur in the yellow parts of the stigma and style. Saffron chromoplasts in red stigma of open flowers are roundish or elongated with tubules forming an electron-opaque reticulum. Some tubules are dilated and form vesicles at the periphery of the plastid. Among the network of numerous tubules are plastoglobules. All plastids contain one or more electron-transparent regions not positive to PATAg staining. Stigmas at anthesis show chromoplasts resulting in mainly vesicles and plastoglobules and are found close to small or large vacuoles. A few mature chomoplasts show bundles of long parallel membranes crossing or encircling the plastidial area. In red stigmas from flowers open for two days, the chromoplasts have only peripheral vesicles and plastoglobules scattered in an electron-transparent stroma. In these cells small or large vesicles in central vacuoles near to the tonoplast membrane are also detected. Chromoplasts in dried stigmas show mainly plastoglobules and empty vesicles spread in plastid, whereas in decoloured stigmas only small plastoglobules, a few vesicles and short thin membranes are observed. On the stigma surface are yellow-coloured papillae, different from the red colour of the stigma. Papillae showed a very large central vacuole and scarce peripheral cytoplasm in which numerous chromoplasts with tubulous reticulum and plastoglobules occur. In the cytoplasm surrounding these chromoplasts there are always vescicles apparently empty except for small vacuoles adhering to the plastids. The chromoplast structure of papillae is similar to that of the plastid present in the yellow part of style. In this part of the style there are plastids with some small starch granules, large plastoglobules and extended system of tubules which at the periphery of the plastid are dilated and form enlarged

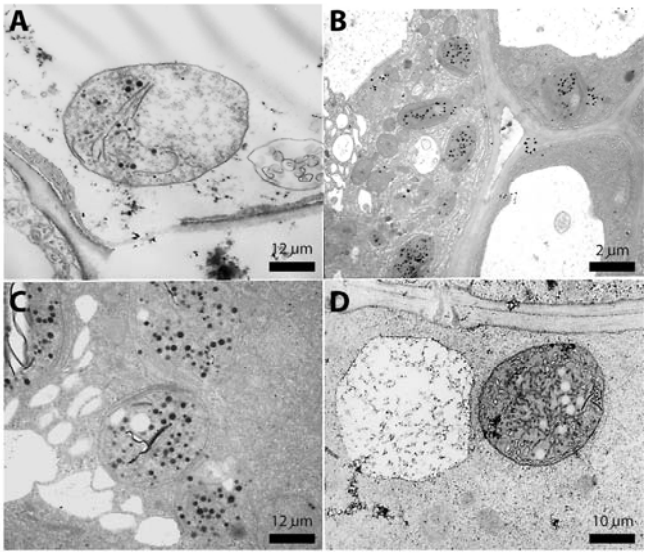

Fig. 8 Chromoplasts (**A**) from stigma of *C. sativus*; (**B**) Cells with chromoplasts from stigma of *C. cartwrightianus*; (**C**) *C. cartwrightianus*; (**D**) *C. thomasii*.

vesicles. In the stroma of these plastids one or more electrontransparent areas are always present. A comparison of plastids from the white part of style, ovary, corm and leaf indicated that plastids with a structure similar to that in chromoplasts or red and yellow part of stigma and style are never present. In the leaf plastids only some plastoglobules occur together with starch granules and tylakoids. Thus the particular structure of saffron red stigma is the presence of tubules, plastoglobules and vesicles. Comparing the ultrastructure of chromoplast of fresh to dried and untreated stigmas to those after extraction of water soluble pigment, we can conclude that apocarotenoids are localized in the tubules and plastoglobules whereas the vesicles can be the site of glycosylate derivatives of crocetin. Tubules in fact disappear in plastids of decoloured stigmas. By contrast plastoglobules, which contain mainly neutral lipids and carotenoids (Deruère *et al*. 1994), persist in all these organelles as well in chloroplasts. They are also widespread in the green and yellow leaves of saffron. In addition, the chromoplast membranes have a high permeability to allow the diffusion of overproduced pigment into cytoplasm (Emes and Tobin 1993) or in autophagic vacuoles (Mesquita 1972). Finally, the water soluble pigment crocetin can be accumulated in the central vacuole of the cell as already hypothesized by Bouvier *et al.* (2003) and Dufesne *et al.* (1999).

C. cartwrightianus chromoplasts (**Fig. 8C**) show many plastoglobules, tubules and one or more electron dense body, spiral like, often crossing the plastid similar to crystal body occurring in carrot roots (Trabucchi 1964) and in tomato fruits (Grilli 1965). *C. thomasii* chromoplasts (**Fig. 8D**) have mainly plastoglobule electrontransparent vesicles encircled in a thin envelope, some thin fibrils and characteristic tubules heavy twisted.

The comparison of chromoplasts of the *Crocus* species related to *C. sativus* suggests that despite the similarity is the basic organization of these plastids, however each species has some different elements and on the whole chromoplasts of *C. sativus* prove to be similar to those of *C. cartwrightianus* and *C. thomasii*.

Parallel to the study of chromoplast structure an analysis of pigment of stigmas of *C. sativus*, *C. cartwrightianus*, *C. thomasii* was carried out in order to detect possible differences in pigment quality and composition amount. Among the pigments separate from crocin, crocetin, picrocrocin and safranal we also analysed the presence of lycopene. This pigment is usually considered to be localized within the chromoplast as tubules due to the thickening of the intraplastidial membranes (Grilli 1965). The preliminary

data indicate that pigment composition and amount are very similar in *C. sativus* and *C. cartwrightianus*. Not lycopene has been detected in stigmas of flowers at anthesis.

CONCLUSION

The data obtained to date from archeology, systematic, cytology, molecular biology, physiology and biochemistry are still insufficient to establish a precise place and moment for the origin of saffron or its parents. However, the information accumulated in recent years offers further indications about the probability that one of the diploid *Crocus* is the parent of *C. sativus*. All data agree that the most probable parent of saffron is *C. cartwrightianus*, only if the origin of saffron is considered by autotriploidy or together with *C. thomasii*, if the origin is due to allotriploidy. In fact:

- remote archeologist and historical records suggest that a *Crocus* has been known and widely used since pre-Hellenistic and Hellenistic times. All authors agree on considering *C. cartwrightianus* as the crocus used in therapy and other fields; wild and cultivated forms are already known;
- saffron grown and used today corresponds to *C. sativus* which morphologically is very similar to *C. thomasii* with which it has been confused in the past;
- *C. sativus* is a triploid species with a karyotype similar to diploid *C. thomasii* and *C. cartwrighitanus*;
- geographic distribution indicates that *C. sativus* and the wild diploid *C.cartwrightianus* and *C. thomasii* occupy common Mediterranean areas;
- biochemical and molecular biology show that DNA of *C. sativus* is most similar to that of *C. cartwrightianus* and not so distant from that of *C. thomasii*;
- reproductive biology makes it possible to ascertain that saffron is self- and allo sterile, although its ovules can be fertilized by pollen of *C. cartwrightianus* as well of *C. thomasii*. The crossing leads to seed set and fruits;
- seeds from crossing saffron with *C. cartwrightianus* are viable and capable of germinating, giving rise to a seedling and then a plant from which new corms originate. The germination and growth behaviour of saffron seeds are very similar to that of *C. cartwrightianus*;
- the chromoplast structure and pigment composition place saffron very close to *C. thomasii* and *C. cartwrightianus*;
- although the path of origin of saffron is still an open question, important new contributions could be reached by new research methods on chromosome structure and molecular genetics. The need now is to research saffron's biological aspects, mainly on crossing with the diploid allied species and then examined the progenies with improved molecular methods. It will be a very long way but we think that a young research team could meet this goal and reach interesting results in order to solve the puzzle of parents of saffron. The results could be useful to obtain genetic amelioration of the cultivated saffron.

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How to reference: Author name(s) (2010) Title of chapter. In: Husaini AM (Ed) Saffron. Global Science Books, UK, pp. X-XX

How to reference: *Sharma KD, Piqueras A (2010) Saffron (Crocus sativus L.) Tissue Culture: Micropropagation and Secondary Metabolite Production. In: Husaini AM (Ed) Saffron. Global Science Books, UK, pp. 15-24*

Saffron (*Crocus sativus* L.) Tissue Culture: Micropropagation and Secondary Metabolite Production

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ABSTRACT

Saffron (*Crocus sativus* L.) is used as a spice, a dye and as a traditional medicine. It is a sterile geophyte and is propagated vegetatively through daughter corms. To meet the steady increase in worldwide demand of saffron, there is a need to expand area under its cultivation, however, limited availability of daughter corms is one of the major handicaps for the expansion of acreage under saffron. Alternatively, micropropagation of saffron using direct or indirect shoot induction or plantlet regeneration through somatic embryogenesis followed by microcorm production offers the capability to produce large quantities of propagating material in short duration of time, however, the protocols available so far need refinement for their commercial utilization. Alternatively, the spice saffron or its chemical constituents viz., crocin, picrocrocin, crocetin and safranal can be produced in tissue cultures. The structures similar to saffron stigmas called as stigma-like structures (SLS) have been generated *in vitro*. Tissue culture-derived SLS have a chemical composition and physical structure similar to natural stigmas; however, lack of their continuous production in tissue cultures is a major bottleneck to exploit this technology at commercial scale. The cell cultures of saffron also synthesize chemical constituents of stigma albeit at lower concentration. Among the four chemicals, production of crocin in cell cultures has been the main focus of research primarily because this chemical is implicated to have anticancer properties. Appropriate concentrations of growth regulators, media components, heavy metals, and two-stage culture system are some of the factors which offer potential to increase production of crocin in cell cultures.

Keywords: cell culture, microcorms, organogenesis, somatic embryogenesis, stigma-like structures, tissue-cultured stigmas

CONTENTS

INTRODUCTION

Saffron is the most expensive spice in the world. This spice is made from the dried stigmas of *Crocus sativus* L. which is a sterile triploid geophyte (2n = 3x = 24). *C. sativus* is propagated vegetatively by means of corms (Fernández 2004). The low rate of production of daughter corms limits the availability of propagating material in saffron. The daughter corms usually take three to four years to mature and give rise to the next progeny of daughter corms. Saffron plants worldwide are considered to be genetically uniform. Because of its narrow genetic base and autotriploid nature, saffron improvement by breeding is very difficult; however, large-sized corms produce more flowers and larger daughter corms (Agayev *et al*. 2009). Thus, there is a possibility of selection of superior clones for higher stigma yield. The multiplication of superior clones in adequate quantities for planting in larger areas is difficult as limited daughter corms are produced by mother corms. Alternatively, daughter corms or plantlets can be propagated rapidly through clonal propagation (Aguero and Tizio 1994; Sharma *et al*. 2008) or embryogenesis in callus cultures followed by generation of shoots/plantlets and daughter corms (Blazquez *et al*. 2004a).

Commercial value of saffron is due to its stigmas which are used as a spice. It is one of the most valuable spice and is recognized for its unique colour, aroma, taste and medicinal properties. The chemicals responsible for spicy properties of saffron are crocin, picrocrocin, crocetin and safranal. These chemicals, especially crocin, also have anticancer properties (Abdullaev 2004). Due to low productivity of saffron (4.6 kg/ha in Iran), limited cultivation in a few countries of the world and decrease in saffron production in all countries of the world in more recent years (Fernandez 2007), the possibilities of increasing saffron production seems to be remote. Another factor that contributes to high-

er costs of saffron is the manual harvesting of stigmas. Tissue culture offers two alternatives to saffron production *in vivo* i.e. generation of saffron stigmas in cultures and production of chemical constituents of saffron in callus or cell cultures (Loskutov *et al.* 1999; Chen *et al.* 2003). Both the techniques have the capabilities for commercialization. In this review, the term metabolite refers to metabolites of saffron stigmas namely crocin, picrocrocin, crocetin and safranal, if not mentioned otherwise.

Research of saffron tissue culture has been reviewed by Plessner and Ziv (1999). A brief account of significant developments in saffron tissue culture has also been presented by Ascough *et al*. (2009) while reviewing micropropagation of Iridaceae. In this review, we have presented an updated account of the research developments on saffron micropropagation, generation of stigma-like structures (SLS) and biosynthesis of important metabolites of saffron stigmas in cell cultures.

PLANTLET REGENERATION FROM PROTOPLASTS

There is only one study on plantlet/shoot generation from isolated protoplasts of saffron (Isa *et al*. 1990). Saffron protoplasts were isolated from calli developed from apical buds or corms (Isa *et al.* 1990; Darvishi *et al*. 2007). A cocktail of two cellulases (1% Cellulase R-10 from *Trichoderma viride* and 1% Driselase from *Irpex lactes*) supplemented with low concentrations of a pectinase, pectlyase Y-23 and 0.3 M Mannitol at pH 5.7 was used as digestion mixture that released sufficiently higher number of protoplasts from callus cultures when treated at 25°C under dark for 1-3 h. Darvishi *et al*. (2007) also used 0.1% MES (2-*N*-morpholino ethane sulfonic acid) in the digestion mixture. Callus cultures treated for 3 h yielded 40×10^5 protoplasts per ml of the suspension with 98% viability (Darvishi *et al*. 2007). After isolation, subsequent growth and division was influenced by protoplast immobilization, nurse cultures and plating density (Isa *et al*. 1990). While protoplasts which were not immobilized in Ca-alginate beads did not divide, the ones embedded in Ca-alginate formed cell clusters with or without nurse cultures. The nurse cultures improved cell division from protoplasts as considerably more number (15%) of cell colonies were formed after two months than protoplasts without nurse cultures (3% cell colonies after two months) (Isa *et al*. 1990). The optimum density of protoplasts in medium for higher frequency of colony formation was 5×10^4 protoplasts per ml. The beads upon transfer to MS medium supplemented with benzyl adenine (BA) and 1-naphthaleneacetic acid (NAA) regenerated shoots and roots i.e., plantlets. About 80% of the calli regenerated on this medium.

The same strategy was used by Karamian and Ebrahimzadeh (2001) to regenerate plantlets from another species of *Crocus* i.e. *C. cancellatus*. Embryogenic callus of *C. cancellatus* initiated from shoot meristems at 17.8 μM kinetin and 4.4 μM 2,4 dichlorophenoxy acetic acid (2,4-D) was used for protoplast culture. Immobilization of protoplasts in Ca-alginate beads followed by 'nurse culture' with 4.4 μM 2,4-D, 8.9 μM kinetin and 0.57 mM ascorbic acid in the dark led to highest growth and cell division. Cells further divided to form cell colonies and embryogenic calli that regenerated plantlets (Karamian and Ebrahimzadeh 2001).

SOMATIC EMBRYOGENESIS

Micropropagation of several plant species has been achieved by callus induction followed by somatic embryogenesis or organogenesis. Among the two modes of plantlet regeneration, embryogenesis is preferred over organogenesis primarily because of the higher frequency of regeneration. The first report in saffron on successful induction of callus and regeneration of intact plantlets was from corm explants (Ding *et al.* 1979, 1981). This was achieved by using culture media supplemented with indole-3-acetic acid (IAA) and 2,4-D. Ilahi *et al.* (1987) also produced callus from corm explants on 2,4-D-containing media followed by bud and shoot differentiation. Different kinds of explants such as corms, corm pieces, apical and axillary buds, young ovaries, young leaves and shoot apices induce callus in saffron (Isa and Ogasawara 1988; Bhagyalakshmi 1999; Blazquez *et al*. 2004a; Sharma *et al*. 2005; Sharifi *et al*. 2010). These explants dedifferentiate to either embryogenic or non-embryogenic calli or mixture of embryogenic as well as non-embryogenic calli. Unlike many crop species where 2,4-D is essential for callus induction, callus in saffron can be induced in the absence of 2,4-D from wide variety of explants such as young ovaries, young leaves, shoot apices and vegetative buds (Igarashi and Yuasa 1994; Sharma *et al*. 2005). In the absence of 2,4-D, growth regulators NAA (auxin) and BA (cytokinin) together in an appropriate ratio induce calli with high efficiency.

Induction of somatic embryogenic callus from non-embryogenic one (explant: meristematic regions of corm) was first reported by George *et al.* (1992). The non-embryogenic callus developed on 2,4-D was transferred to medium containing IAA, kinetin and ascorbic acid where somatic embryo development took place. This was followed by induction of embryogenic callus from bulblet explants in the presence of growth regulators BA and NAA (Ahuja *et al.* 1994). With the advent of somatic embryogenesis in saffron, role of 2,4-D and other growth regulators in embryogenesis became clear. 2,4-D is required for induction of embryogenic callus whereas its absence lead to the development of non-embryogenic calli (Karamian 2004; Sharma *et al*. 2005; Darvishi *et al*. 2007; Raja *et al*. 2007). 2,4-D alone, however, is not very effective in embryo induction and supplementation with kinetin or BA along with 2,4-D is essential for high frequency of induction. For example, embryogenic callus developed from shoot meristems in the presence of 4 mg L^{-1} kinetin and 1 mg L^{-1} 2,4-D (Karamian 2004) and that from shoot and leaf explants in the presence of 2,4-D and BA (Raja *et al.* 2007). In addition, jasmonic acid (Blazquez *et al.* 2004b) and thidiazuron (TDZ) (Sheibani *et al.* 2007) also improve efficiency of somatic embryogenesis. Compared to other hormones, low amounts of TDZ are required for embryo induction (0.5 mg L^{-1}) and proliferation (0.25 mg L^{-1}). Generally, whole of the callus does not convert uniformly to embryos and only some regions form embryos. These regions are called as embryo-rich regions and are selectively multiplied during subsequent subcultures to get enough embryos (Blazquez *et al.* 2009). Factors affecting maturation of embryos in saffron have not been studied in detail except that elevated levels of sucrose (6%) in hormone-free MS (Murashige and Skoog 1962) medium (Sheibani *et al.* 2007) or 1 mg L^{-1} abscisic acid lead to maturation of embryos (Karamian 2004). Mature embryos were germinated on 25 mg L^{-1} gibberellic acid, GA_3 (Karamian 2004). Upon germination, the basal parts of the embryos usually swell leading to the formation of microcorms after 3 months (Sheibani *et al.* 2007). Rapid increase in cell or callus mass is desirable to achieve sufficiently higher amounts of embryos. The use of a temporary immersion system increases fresh weight of embryogenic calli four times compared to those grown on solid media (Blazquez *et al.* 2004b).

The embryogenic calli look nodular. It takes about 6 weeks to get nodular embryogenic calli from corm tissue cultures (Blazquez *et al.* 2009). At the nodular stage, the calli contain proembryonic structures or proglobular embryos which develop to globular embryos after 3 weeks in culture, to monopolar (containing a meristem and cotyledon) after 7 weeks in culture and to bipolar embryo (consisting of an apical meristem with a cotyledon at one end and minicorm at other end) after 10 weeks in culture (**Fig. 1**, Blazquez *et al.* 2009). It takes about two weeks in culture for maturation of bipolar somatic embryos. The development of the globular structure of embryos is coupled to the concomitant development of protodermis which is the outermost layer of the developing embryo and has clinical

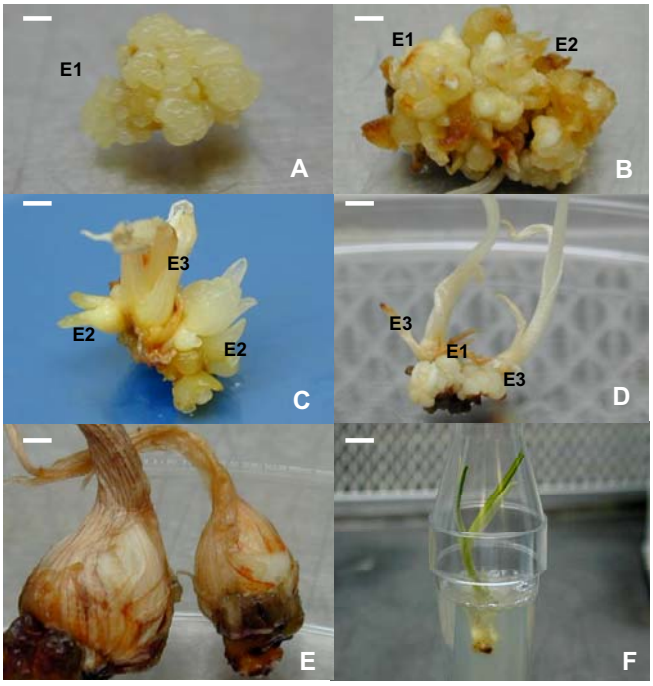

Fig. 1 Somatic embryogenesis in saffron. **A**, **B**, **C** and **D** show embryogenic calli with somatic embryos at different developmental stages (**E1**: globular, **E2**: monopolar, **E3**: dipolar). **E** presents a fully developed somatic embryo with a minicorm and **F** is a plantlet developed from an **E3** somatic embryo. The scale bar is 0.5 cm in **A**, **B**, **C** and **D** and 1 cm in **E** and **F**. (Blazquez 2004c).

divisions. The presence of this layer is considered as an evidence or characteristic feature of somatic embryo development (Von Arnold *et al.* 2002). The protodermis applies physical and cell divisional limitations and regulates further developments (Quiroz-Figueroa *et al.* 2002). The protodermis can be seen in histological sections during early stages of embryogenesis in saffron and embryogenesis follows the normal path (Blazquez *et al.* 2009) similar to other genera and species such as *Iris* (Wang *et al.* 1999), *Gladiolus* (Stefaniak 1994) and *Allium sativum* (Fereol *et al*. 2002). Apart from callus cultures, parthenocarpic fruits of saffron developed *in vitro* also develop embryos (Chichiricco and Grilli Caiola 1987), however, development stages of these embryos are not studied and hence, similarities or differences in the development of embryos in cells and parthenocarpic fruits are not known.

Like other plant species, embryogenesis in saffron is accompanied with cellular stresses (Blazquez *et al.* 2004a). Plant response to stress usually leads to accumulation of reactive oxygen species (ROS). In cell cultures, ROS such as hydrogen peroxide (H_2O_2) are generated during induction of embryogenesis and are suspected to play role in regenerative pathways in plant tissue cultures including embryo induction (Kairong *et al.* 1999; Tian *et al*. 2003). More specifically, H_2O_2 in the presence of ascorbate glutathione maintains cell wall plasticity and stimulate organized cell growth (De Gara *et al*. 1997). In several plant species, the role of ROS appears to be confined to early stages of somatic embryogenesis and these might not be required for subsequent development of the embryos. Saffron seems to be no exception. The activities of ROS, enzymes of the antioxidant system (ascorbate peroxidase, dehydroascorbic acid reductase and glutathione reductase) and catalase increase considerably during somatic embryogenesis in saffron (Blazquez *et al.* 2004a, 2009). Several members of the superoxide dismutase (SOD) family, the enzymes involved in stress tolerance in plants also play role in embryogenesis. Two Mn-SOD and four Cu, Zn-SODs are involved in embryogenesis while Mn-SODs increase during last two stages of embryogenesis and Cu, Zn-SODs decrease (Blazquez *et al*. 2009). In saffron, stress most probably the oxidative one followed by ROS appear to function as components of signal transduction chain required to reprogram gene expression and induce totipotency to gain embryogenic competence by the somatic cells (Blazquez *et al.* 2004a, 2009). The genes involved in embryogenesis in saffron are not known, however, based on small cDNA library prepared from corms, some genes with probable role in regulation of embryogenesis *in vitro* were identified (Alvarez-Orti *et al.* 2004). While constitutive genes showed similar expression patterns at all stages, developmentally regulated genes changed their expression. Expression of a xyloglucan endotransglycosylase [xyloglucan:xyloglucosyl transferase] (a cell wall loosening activity enzyme involved in cell growth), formaldehyde dehydrogenase and an abscisic stress ripening protein was strongly regulated.

The shoots/plantlets from callus cultures develop in the presence of cytokinins (BA, kinetin) either alone or in combination with auxins such as indole-3-butyric acid (IBA), NAA (Ahuja *et al.* 1994; Igarashi and Yuasa 1994; Sharma *et al.* 2005). As expected, 2,4-D inhibits shoot induction. Shoot/plantlet development was studied from both embryogenic and non-embryogenic calli and in both the cases growth regulators were used for shoot induction (from non-embryogenic calli) and plantlet development (embryogenic callus, George *et al*. 1992; Sharma *et al*. 2005). Type of nitrogen source also effects shoot regeneration from non-embryogenic calli. Nitrogen in the form of nitrate (NO_3) favours induction of shoots whereas the ammonical (NH_4^+) form of nitrogen has inhibitory effect (Igarashi and Yuasa 1994).

The cost of production of saffron shoots *in vitro* either through embryogenesis or organogenesis is high and alternatives are needed to lower the cost of their production. The major cost enhancing factors in shoot regeneration are labour, sucrose and agar. While the labour costs could not be reduced until the tissue culture operations are performed by using the robots, the amount of sucrose in media could be reduced by using photoautotrphic micropropagation under high light and CO_2 intensity especially after induction of shoots, and agar omitted from the media by using liquid cultures. By using cotton bed as a substitute of agar, the cost of shoot induction and development could be reduced by 33.5% (Sharma *et al.* 2005); however, the frequency of shoot regeneration was low in media devoid of agar. Probably agar altered nutrient availability and uptake by saffron cells as its type and concentration in medium is known to alter nutrient uptake and influence shoot development in tissue cultures (Beruto *et al.* 1999; Karim *et al.* 2003).

DIRECT SHOOT REGENERATION FROM DIFFERENT EXPLANTS

Somatic embryogenesis in saffron is not very efficient and plantlet generation from somatic embryos is low. These problems must be resolved before somatic embryogenesis can become a viable method for mass propagation of saffron. Direct shoot regeneration without an intervening callus pahse offers an alternative to somatic embryogenesis. The direct shoots have been generated from apical buds, lateral buds, small corms and ovaries (Plessner *et al.* 1990; Aguero and Tizio 1994; Bhagyalakshmi 1999; Sharma *et al.* 2008; see **Fig. 2** for direct shoots generated from apical buds). The direct organogenesis has the advantage of more genetic uniformity compared to adventitious regeneration from callus cultures or somatic embryogenesiss (Piqueras and Debergh 1999). Usually less time is required to generate direct shoots compared to indirect ones.

Efficacy of direct shoot regeneration in saffron depends upon the explant used, growth regulator composition and temperature of incubation. First report of direct shoot regeneration was from corms (Homes *et al*. 1987) that was followed by shoot regeneration from apical buds consisting of 2 mm sided cube of corm tissue (Plessner *et al.* 1990). Shoot induction from apical buds was promoted by cytokinins (kinetin or zeatin) and temperature of 15 or 20°C

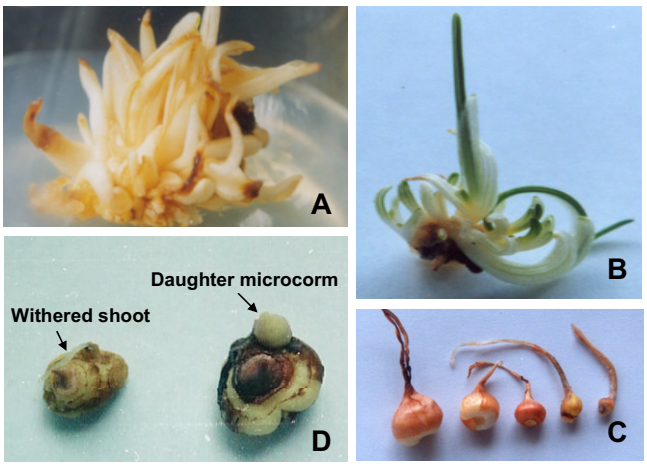

Fig. 2 Multiple shoot and microcorm generation from bud explants *in vitro* in *Crocus sativus*. (**A**) Direct multiple shoots induction; (**B**) green shoots; (**C**) mature microcorms; (**D**) microcorms bearing daughter microcorms. Microcorms first germinated (withered shoot is visible at the top of microcorm on the left hand side) followed by swelling at the base of shoots leading to daughter microcorm formation.

(Plessner *et al.* 1990). Among different cytokinins, BA is the most effective cytokinin in direct shoot induction from buds and small corms (Majourhat *et al.* 2007; Sharma *et al.* 2008). A recent study indicated that TDZ at concentrations below 10 µM is far more effective than BA in shoot induction from apical buds (Sharifi *et al.* 2010). Most characteristic feature of the shoot induction using TDZ is an intermediate nodular or embryo-like structures which lack root pole (Sharfi *et al.* 2010). Sucrose concentration is another factor that influences multiple shoot regeneration from corms as shoot induction is more at higher concentration (50 g L^{-1}) compared to lower one (30 g L^{-1}) (Sharma *et al.* 2008). The effect of sucrose is not unique in saffron as sugar concentrations are known to affect shoot and root regeneration in many plant species (Radhika *et al.* 2006; Soniya and Sujatha 2006; Novotna *et al.* 2007).

As for buds and corms, BA is also the best growth regulator for shoot induction from ovaries, however, shoot induction from ovaries is favoured by lower concentrations of BA compared to those required for direct shoot regeneration from corms or buds. At concentrations comparable to those used for shoot induction from buds or corms, it induced either calli or abnormal shoots from ovaries. The optimum concentration of BA for shoot induction from ovaries is 4.4 µM along with higher NAA (26.9 µM) concentration (Bhagyalakshmi 1999). Apart from growth regulators, growth stages of ovaries also influence shoot induction and ovaries with yellow stigma are most amenable (Bhagyalakshmi 1999).

Saffron ovaries have been used to generate calli, shoots and stigma-like structures (SLS) and ratio of BA to NAA seems to be the most critical factor leading to organogenesis or dedifferentiation. Absence or low levels of IBA or NAA along with BA lead to direct shoot induction from ovaries as well as other explants (see above; Darvishi *et al.* 2007; Sharma *et al.* 2008) whereas low concentrations of NAA (5.4 µM) and higher ones of BA (44. 4 µM) induce SLS from half ovaries (Loskutov *et al.* 1999, **Table 1**). A 1:2 ratio of these growth regulators (BA 5 mg L^{-1} and NAA 10 mg L^{-1}) supports callus induction (Castellar and Iborra 1997). Interactions among growth regulators, however, appear to be far more complex as equal ratio of both NAA (1 mg L^{-1}) and BA (1 mg L^{-1}) also differentiated ovaries into SLS (Castellar and Iborra 1997; Loskutov *et al.* 1999).

Temperature and light influence direct shoot induction from explants. Usually temperature around 20°C is best for shoot induction and development; however, 25°C also supported induction and development of shoots (Plessner *et al.* 1990; Bhagyalakshmi 1999). While the effect of light on shoot induction form buds/corms has not been studied, dark conditions enhance shoot bud induction from ovaries (Bhagyalakshmi 1999). Like embryogenesis, the changes in activities of antioxidative enzymes such as peroxidase, catalase, SOD, esterase and polyphenoloxidase were also noticed during shoot induction (Sharifi and Ebrahimzedah 2010). It appears that antioxidant enzymes play a major role in the process of organogenesis and embryogenesis in saffron.

Efficient micropropagation of saffron can not be achieved unless tissue culture derived shoots are multiplied *in vitro*. There is only one study (Sharma *et al.* 2008) on this aspect where individual *in vitro* derived shoots developed multiple shoots at low frequency. Maximum number of shoots (4.0) per cultured shoot induced at 14 mg L^{-1} BA + 3 mg L^{-1} IBA + 50 g L^{-1} sucrose was, however low.

IN VITRO MICROCORM PRODUCTION

Natural way of propagation of saffron is through cormels called as cormlets which develop on the mother corms. The cormlets grow in size while in soil and after attaining optimum size (three to four years) give rise to next generation of cormlets. During shoot/plantlet development experiments, it has been found that *in vitro* developed shoots have tendency to swell at the base followed by formation of the small cormlet also called as microcorm (**Fig. 2**, Gui *et al.* 1988; Plessner *et al.* 1990; Plessner and Ziv 1999; Sharma *et al.* 2008). The microcorms *in vitro* can be produced in large numbers in short duration of time and are considered ideal for saffron micropropagation. Being smaller in size, these are easy to transport and store under low temperature conditions and can be used for germplasm storage. However, generation of large number of microcorms for commercial use in saffron is elusive so far. The induction of microcorms from directly generated shoots was achieved for the first time by Plessner *et al.* (1990). The corms with adventitious shoots when rooted in medium without growth regulators also give rise to microcorms. Given appropriate culture condition, the tissue culture derived shoots form microcorms

Table 1 Effect of BA and NAA (mg l^{-1}) for callogenesis and organogenesis from ovaries, apical buds and corms of saffron.

Explant	BA	NAA	Organ induced	Reference
Ovary	5.0	10.0	callus	Castellar and Iborra 1997
Ovary	5.0	4.0	Shoots	Bhagyalakshmi 1999
Ovary	1.0	5.0	Shoot development	Bhagyalakshmi 1999
Ovary	1.0	10.0	SLS	Sarma *et al.* 1991
Ovary	1.0	1.0	SLS	Castellar and Iborra 1997
Half Ovary	10.0	1.0	SLS	Loskutov *et al.* 1999
Apical bud[a]	6.0	0	Shoots	Sharma *et al.* 2008
Corms	6.0	0	Shoots	Sharma *et al.* 2008
Apical bud	2.0	2.0	Non-embryogenic callus	Darvishi *et al.* 2007
Apical bud	1.0	[b]	Embryogenic callus	Darvishi *et al.* 2007
Apical bud	0	[c]	Callus and shoots	Sharifi *et al.* 2010

[a]Also called as apical meristem and consists of an apical bud along with about 2 mm or larger sided cube of corm tissue.
[b]1.0 mgl^{-1} 2,4-D
[c]1.0 mgl^{-1} TDZ

irrespective of the explant used to generate shoots. The shoots derived from ovaries, floral and corm segments as well as plantlets developed from somatic embryos from leaf explants form microcorms (Bhagyalakshmi *et al.* 1999; Karaoglu *et al.* 2007; Raja *et al.* 2007).

Ethylene treatment, microsurgery of the apical bud, concentration of media salts, growth regulators and growth retardants such as paclobutrazol and imazalil favour microcorm induction (Plessner *et al.* 1990; Piqueras *et al.* 1999; Sharma *et al.* 2008). The process of induction and development of microcorms is an energy requiring process and deposition of biomass at the base of the shoot is affected by sucrose concentration (Sharma *et al.* 2008). In saffron as well as in other crop species (Chow *et al.* 1992; Madubanya *et al.* 2006), sucrose/carbohydrate concentration is the most critical factor for cormogenesis and higher sucrose concentrations (6-9%) favoured microcorm induction and development (Aguero and Tizio 1994; Raja *et al.* 2007; Sharma *et al.* 2008). Sucrose, however, does not have the same affect on storage organ formation in all the crops. It affects number and size of storage organs differently in different crops, e.g. increase in sucrose concentration from 3-9% decreases microcorm induction frequency but increases corm mass in *Watsonia vanderspuyiae* (Ascough *et al.* 2008), in *Lachenalia* increase from 3 to 6% does not improve bulblet formation but does increase size (Slabbert and Niederwieser 1999). Saffron does not falls in both of these categories as higher concentrations of sucrose increase both the microcorm induction as well as their average mass. Sucrose is essential for microcorm induction as media devoid of sucrose form no microcorms whereas frequency of induction deceases considerably (0.29 per shoot) when mannitol (1.89 per shoot on medium containing comparable sucrose), a sugar alcohol that is not metabolized by plant tissue, is used as sole carbon source (Sharma *et al.* 2008). Sucrose is considered by many as an osmolyte that increases stress leading to storage organ induction, however, in saffron role of sucrose appears be more than osmolarity. No microcorm formation in the absence of sucrose, very little in the presence of mannitol, point that sucrose might be providing energy for corm induction and growth. Like saffron, addition of mannitol or sorbitol at concentrations comparable to sucrose in *Narcissus* 'St. Keverne and Hawera' does not stimulate bulblet formation (Staikidou *et al.* 2005). The type of carbohydrate source may also affect storage organ induction e.g. in *Hyacinth*, fructose is more effective than glucose or sucrose in bulblet induction (Bach *et al.* 1992). However, sucrose and mannitol are the only carbohydrates tested so far for microcorm induction in saffron and effect of other carbohydrate sources is not studied.

Lower salt concentrations e.g. half strength of MS salts improves microcorm induction and development compared to full strength of salts (Raja *et al.* 2007; Sharma *et al.* 2008). Growth regulators IBA, BA, NAA and abscisic acid (ABA) affect microcorm induction with 3 mg L^{-1} BA in half-strength MS medium best for induction and development with formation of as many as 1.89 microcorms (1.18 g average weight) per shoot (Sharma *et al.* 2008). BA alone or in combination with other growth regulators is the most important growth regulator for cormogenesis (Piqueras *et al.* 1999; Sharma *et al.* 2008). BA induces storage organ formation in many crops such as *Gladiolus* (Steinitz *et al.* 1991; Ziv 1992), and *Crinum* (Slabbert *et al.* 1993). Fundamental differences appear to exist in different plant species for transmission of signals for induction of storage organs. In some crops, storage organ formation is induced by BA and anti-gibberellin compounds such as paclobutrazol whereas GA_3 inhibit their induction. In other crops like *Watsonia vanderspuyiae* (Ascough *et al.* 2008) and *Dierama luteoalbidum* (Madubanya *et al.* 2006), GA_3 increased cormogenesis. The role of GA_3 in microcorm inhibition or induction in saffron is not evaluated, and at the same time, role of growth retardants in corm induction is not established conclusively; though, there is an indication that paclobutrazol and imazalil increase growth of microcorms (Piqueras *et al.* 1999). Thus, saffron appears to fall in the first category where GA_3 inhibits cormogenesis. ABA which is associated with senescence inhibits corm induction and development as well as fructification leading to development of parthenocarpic fruits in saffron (Chichiricco and Caiola 1987; Sharma *et al.* 2008). The inhibitory effect of ABA on corm induction has also been reported in *Watsonia vanderspuyiae* (Ascough *et al.* 2008).

The type of explant also appears to affect cormogenesis. While individual shoots show poor cormogenic response, bunches of two to three shoots develop more number of microcorms having higher average weight (Sharma *et al.* 2008). The period of harvest of corms for explant isolation and season of culture can affect corm development as the seasonal developmental cycles typical for saffron in a natural environment do not change during *in vitro* culture (Milyaeva *et al.* 1995). The optimum temperature for corm development *in vivo* under phytotron conditions is 17°C day/ 12°C night with 27°C day/22°C night the highest for corm development (Plessner 1989). A wide variety of temperatures (10 to 25°C) were tested for microcorm induction *in vitro* (Plessner *et al.* 1990; Milyaeva *et al.* 1995; Bhagyalakshmi 1999; Sharma *et al.* 2008) and 20°C is the optimum for cormogenesis *in vitro*. Synergistic interactions between temperature and growth regulators for corm induction may also occur as has been observed in *Watsonia vanderspuyiae* (Ascough *et al.* 2008). The role of light in corm formation in saffron is also not clear as partial or continuous light has been used to induce corms from shoots (Plessner *et al.* 1990; Milyaeva *et al.* 1995; Sharma *et al.* 2008). Light affects storage organ formation differently in different crops. In some species, storage organ induction is inhibited by continuous dark e.g. *Lilium* (Lian *et al.* 2003), *Fritillaria* (Paek and Murthy 2002), *Watsonia vanderspuyiae* (Ascough *et al.* 2008), whereas in others it is either favoured by dark e.g. *Narcissus* (Steinitz and Yahel 1982) or not influenced by light (16 h photoperiod) or continuous dark e.g. *Hyacinthus* (Kim *et al.* 1981), *Lachenalia* (Slabbert and Niederwieser 1999). In saffron, information on the effect of both temperature and light on microcorm formation is limited and more research is needed to establish optimum temperature, light and interactions among temperature, light and growth regulators, if any for cormogenesis.

One of the recent protocols for microcorm induction from shoots (Sharma *et al.* 2008) is outlined in **Fig. 2**. Using this protocol, it took nine months to develop microcorms (1.89 microcorms per shoot, 1.18 g average weight) from culture of buds. The microcorms were comparable in shape and size to daughter corms obtained under field conditions (1.2 g average weight) after 22 months (Chahota *et al.* 2003). The majority (95.6%) of microcorms sprouted *in vitro* on MS medium supplemented with growth regulators and two of the cold pretreated (4°C for 4 months) microcorms (out of 10) also developed daughter microcorms. Sprouting and daughter microcorm differentiation was similar to daughter corm production under field conditions. The performance of these microcorms, however, was not evaluated under field conditions. It has been established that larger corms give rise to larger and more number of cormlets *in vivo* that bear more number of flowers (Gresta *et al.* 2008; Agayev *et al.* 2009). Larger corms also bear more flowers and yield more (Cavusoglu *et al.* 2009). Since microcorms vary considerably in size, it would be interesting to see if there exist differences in performance of larger and smaller microcorms under field conditions.

PRODUCTION OF CROCIN, CROCETIN, PICROCROCIN AND OTHER METABOLITES *IN VITRO* BY CALLUS/CELL CULTURES AND STIGMA-LIKE STRUCTURES

Saffron, the dried red stigmas of *C. sativus* L., being used as colouring and flavouring agent and as medicine are in huge demand, but low productivity and decline in area under crop limits availability. Being a labour intensive enterprise

and low average yield, the cost of natural stigmas is too high. Alternative economical ways to produce saffron include tissue culture methods (Loskutov *et al.* 1999). The approach being followed to accomplish this is the generation of saffron (stigmas) in tissue cultures (Loskutov *et al.* 1999; Zeng *et al.* 2003). Production of crocin, crocetin, picrocrocin and safranal, the important chemical constituents of spice saffron in calli or cell suspension cultures without induction of stigmas is another method that can be exploited by the industry. In other plants, colouring agents such as shikonin are being produced commercially in somatic cell cultures (Fujita *et al*. 1982). The tissue culture methods, if exploited, can reduce dependence on natural stigmas and may lower the cost of this spice.

Callus/cell cultures

Induction of secondary metabolites in callus or cell cultures of some plant species has been studied extensively; however, limited information on this aspect is available in saffron. Initial studies indicated that unorganized cells of saffron produced colouring agents like crocin or crocetin, albeit at very low concentrations compared to those produced in the natural or *in vitro* generated stigmas (Hori *et al*. 1988; Visvanath *et al.* 1990; Dufresne *et al.* 1997; Zeng *et al.* 2003). The crocin content in saffron callus was only 0.24% compared to 14.30% in the natural stigmas and up to 6.0% in SLS (Zeng *et al.* 2003). In addition to low amounts of secondary metabolites, saffron callus cultures also suffer from browning during culture (Visvanath *et al.* 1990). The brown parts of the callus are dead/dying cells with no capability to divide or produce secondary metabolites. Initial studies used one-stage culture system for metabolite production. In other plant taxa where cell cultures are used for secondary metabolite production, one-stage culture system is not very efficient because conditions for division and growth of cells usually differ from those for secondary metabolite production. The saffron is no exception as cell growth and crocin formation are separate events (Chen *et al.* 2003) and efficacy of crocin production was considerably more when two-stage culture system was used. In two-stage culture system for saffron, NAA (2.0 mg L^{-1}) and BAP (1.0 mg L^{-1}) in B5 medium (Gamborg *et al*. 1968) supplemented with casein hydrolysate (300 mg L^{-1}) yield maximum biomass whereas IAA (2.0 mg L^{-1}) and BAP (0.5 mg L^{-1}) produce maximum crocin. Using two-stage culture system, crocin production was 43 mg L^{-1} (6.32 mg g^{-1}) i.e. 295% of that of the one-stage culture system. As expected, one of the major factor contributing to higher crocin production in two-stage culture system is the high biomass yield (6.8 g L^{-1}). Unlike SLS, where light and 25°C enhance synthesis of secondary metabolites of saffron, darkness and 22°C is optimum for production of crocin from cell cultures.

Precursors enhance metabolite production in cell cultures considerably, however, impact of precursor feeding on production of secondary metabolites of saffron is not known. Two rare earth elements, La^{3+} and Ce^{3+}, enhance cell growth and crocin production in saffron (Chen *et al.* 2004). While La^{3+} promotes growth of cells with little impact on crocin production, the Ce^{3+} increases production of crocin with little effect on cell growth. Surprisingly, La^{3+} and Ce^{3+} together in the medium increase both the cell biomass (1.7-fold, 20.4 g l^{-1}) as well as crocin production (4.2-fold, 4.4 mg g^{-1}, 90 mg l^{-1}). Though, exact mechanism of action of rare earth elements is not clear, these are expected to react with certain enzymes in the cells and on the membrane and modify enzyme function and cell permeability. This can lead to increased uptake and utilization of nutrients (Guo 1999; Hong *et al.* 1999) and fast growth of saffron cells. The mode of action of these elements might be similar to that of heavy metal ions which are also known to induce production of secondary metabolites at lower concentrations.

Though, limited studies have been conducted so far on crocin production in cell cultures, these might provide leads to the development of bioreactor based systems for commercial production of saffron metabolites in cell suspensions. Further experimentation is needed to standardize factors affecting cell growth and metabolite production in bioreactors. Some of these factors are, heavy metals, precursor chemicals, growth regulators, casein hydrolysae, light and temperature. The studies during the last a few years are only on crocin production, primarily because demand for crocin is expected to rise in near future as this compound has anticancer properties (Escribano *et al.* 1996). Moreover, crocin is synthesized from crocetin, another metabolite of saffron by enzyme glucosyltransferase, and bioconversion of crocetin to crocin is not successful (Dufresne *et al.* 1999).

Saffron corms also contain a proteoglycan that inhibits growth of human tumor cells. This glycoconjugate is cytotoxic against human cervical epithelioid carcinoma cells (IC_{50} = 7 mg mL^{-1}), and consists of approximately 90% carbohydrate and 10% protein. The proteoglycan was also synthesized in callus cultures developed from saffron corm (Escribano *et al.* 1999). Apart from human tumor cells, reversible cytostatic effect of the arabinogalactan protein from saffron on root growth and *in vitro* viability of plant cells has also been documented (Fernández *et al*. 2000). This seems to be the maiden report till date that describes cytotoxicity of proteins of plant origin on plant cells.

Stigma-like structures

The stigmas generated *in vitro* are called as stigma-like structures (SLS) or tissue culture stigmas (TCS). Followed by the first report on *in vitro* proliferation of SLS in *Nicotiana tabacum* (Matsuzaki *et al.* 1984), Sano and Himeno (1987) showed proliferation of young intact stigma plus ovaries, single stigmas and half ovaries of saffron into stigmas under *in vitro* conditions with half ovaries being the best explant (75 SLS per half ovary). The SLS contain crocin, crocetin, picrocrocin and safranal, all the pigments found in natural stigmas. The SLS can be generated from explants either directly or indirectly through meristematic tissue on media containing BA/kinetin and NAA. The generation of SLS in tissue cultures is common and can be achieved from wide variety of explants such as immature ovaries, half ovaries, stigmas, stigma plus ovaries, anthers, stamens and petals and on wide range of media (Himeno and Sano 1987; Sano and Himeno 1987; Namera *et al*. 1987; Fakhrai and Evans 1990; Sarma *et al.* 1990, 1991; Kohda *et al.* 1993; Loskutov *et al.* 1999; Zhao *et al.* 2001); however, frequency of generation of SLS and concentration of chemical constituents in SLS remained low (Fakhrai and Evans 1990; Sarma *et al.* 1990; Sarma *et al.* 1991; Kohda *et al.* 1993). The amount of crocin, and picrocrocin was lesser by 6 and 11 times in SLS compared to that in natural stigmas. In general, SLS were low in floral, spicy and fatty characteristics as compared to saffron obtained from flowers (Sarma *et al.* 1991). In addition to SLS, petal explants also generate petal-like structures (PLS) which develop SLS in frequencies higher than the SLS induced directly from petals (Wang *et al.* 2002).

Sustained proliferation of SLS is essential for commercialization of technology and needs attention for research. Major hurdles in sustained proliferation of SLS *in vitro* are low frequency of generation of SLS, browning of SLS, formation of non-SLS structures and single harvest. Besides sustained proliferation, low concentration of secondary metabolites, slow growth of callus tissues and short life of explants are the other problems. Browning and low rate of SLS production may be correlated as browning renders the cells dead which can otherwise generate SLS. Repeated subcultures at short intervals, use of B5 medium in place of MS medium and addition of activated charcoal in medium allowed three harvests of SLS within a period of 9-10 months (Loskutov *et al.* 1999). In addition to B5 medium and growth regulators, casein hydrolysate (CH) and L-alanine improved further the induction of SLS, and these along with rapid subculture, B5 medium and activated charcoal yielded SLS with content of crocin, crocetin,

picrocrocin and safranal comparable to or even higher than that produced by naturally grown stigmas. L-alanine is a precursor of saffron metabolites (Zeng *et al.* 2003); however, role of CH in enhancing metabolite content is not known. Though, Loskutov *et al.* (1999) improved considerably the SLS production, proliferation beyond three harvests requires further research efforts.

Understanding of the physiology of stigma induction and synthesis of chemical constituents of saffron is in infancy. A vast amount of literature indicates that yields of secondary metabolites increase with addition of the precursors. Compounds like crocin and crocetin are terpenoids (Dufresne *et al.* 1999). All terpenoids originate from acetyl CoA and compounds increasing production of acetyl CoA in cells can enhance production of saffron metabolites in SLS. Based on this assumption, L-alanine, sodium acetate, glycine and serine were used as precursors to enhance crocin production in SLS (Otsuka *et al.* 1992; Zeng *et al.* 2003). These compounds in plant cells are converted to acetyl CoA, albeit using different enzymes. Glycine first converts into serine and then to acetyl CoA. Of the four precursors used, L-alanine and sodium carbonate enhance not only production of SLS but also increase content of crocin, picrocrocin and safranal (Otsuka *et al.* 1992; Zeng *et al.* 2003). The concentration of crocin in SLS (6.0%) produced in the presence of sodium acetate was two fold more compared to the SLS generated on basal medium (2.21% crocin), however, the quantity was less by half compared to natural stigma (14.30% crocin, Zeng *et al.* 2003). The amount of crocin produced in SLS also depends upon light (4.91% on basal medium under light, 2.21% under dark) and polyvinyl pyrrolidone, PVP (5.22%). Despite the effectiveness of light and PVP in enhancing accumulation of crocin, these does not increase SLS induction, however, SLS formed in the presence of PVP were morphologically more like natural stigmas. It would be interesting to see how the combination of sodium acetate, light and PVP would affect SLS induction and accumulation of different metabolites of saffron. Loskutov *et al* (1999) have generated SLS with high concentration of crocin, picrocrocin, crocetin and safranal under dark. Since light enhances crocin accumulation by two times (Zeng *et al.* 2003), it might be possible to have SLS with an increased concentration of saffron metabolites compared to natural stigmas if the method of Loskutov *et al.* (1999) is combined with incubation under continuous light conditions.

Direct or indirect mode of induction of SLS depends upon hormonal composition of the media. While low concentrations of NAA and BA induce direct SLS, high concentrations of these growth regulators induce indirect types (Loskutov *et al.* 1999; Ebrahimzadeh *et al.* 2000, 2001). In general, the direct types are more similar to natural stigmas in shape, colour and size compared to the indirect ones. Similarly, accumulation of crocin, picrocrocin and safranal in direct types is more compared to indirect types (Ebrahimzadeh *et al.* 2001). The direct types also have proportion of different forms of crocetin i.e. monoglucoside esters, diglucoside esters crocetin ester types comparable to natural stigmas.

The systematic studies on affect of temperature on induction and proliferation of SLS are not available. Since, saffron is a crop of colder regions, induction and development of SLS might be thought to be better at lower temperatures. Initial experiments on SLS were also conducted at low (20°C) temperature (Sano and Himano 1987; Sarma *et al.* 1991). Flowering in saffron requires exposure of corms at higher temperatures (25°C) for as many as 55 days (Molina *et al.* 2005). The flowering could be accelerated (up to seven days) by increasing the temperature to 30°C prior to exposure at 25°C. However, elevated temperatures for longer durations might be deleterious for bud growth and flower formation. The optimum temperature for flower formation is 23 to 27°C, 23°C slightly better than 27°C and minimum exposure time of 50 days (Molina *et al.* 2004). Does that mean that the induction and development of SLS would be better at 25°C than that at 20°C? Though, there is no information available on this aspect, the incubation temperature used in later studies on SLS was 25°C or room temperature (Loskutov *et al.* 1999; Zeng *et al.* 2003).

CONCLUSIONS

Considerable progress has been made in saffron micropropagation and SLS formation whereas studies on synthesis of metabolites in cell cultures are limited only to crocin production (**Table 2**). Somatic embryogenesis, as a mode of propagation is still not very efficient because of low rate of induction of embryos in callus/cell cultures. The knowledge on possible role of oxidative stress (Kairong *et al.* 1999; Blazquez *et al*. 2009) and some chemicals (TDZ and NO_3^- form of nitrogen) on improvement of embryogenesis may be useful to enhance embryo induction *in vitro*.

At present, direct shoot induction followed by microcorm formation is an area that holds promise for commercialization especially if genetically improved saffron corms (Agayev *et al.* 2009) are to be multiplied with in a short span of time. There are three areas of concern in this field which are required to be addressed to make this technology viable for micropropagation, i) sustained multiplication of shoots from tissue culture derived shoots, ii) development of large sized microcorms and iii) field evaluation of microcorms for agronomic performance. There is only a report on *in vitro* multiplication of tissue culture derived shoots (Sharma *et al.* 2008) and no report on evaluation of microcorms under field conditions. In addition to this, there is also a need to study effect of light and temperature on shoot induction and microcorm formation because interactions between growth regulators and temperature determine the efficacy of hormonal treatments whereas light either inhibit storage organ formation, or enhance it or has no influence (Steinitz and Yahel 1982; Slabbert and Niederweiser 1999; Lian *et al*. 2003). Physiology of corm induction in saffron is also different from genera like *Watsonia vanderspuyiae* (Ascough *et al*. 2008) and *Dierama luteoalbidum* (Madubanya *et al.* 2006) where GA_3 promotes cormogenesis. In saffron, BA is a signaling molecule for cormogenesis and antigibberellic compounds promotes corm induction. Higher cost of micropropagation in many plants acts as a main hindrance for commercialization. Some of the major contributors of higher cost are sucrose and agar. One of the alternatives to reduce micropropagation cost in saffron is to switch to photoautotrophic micropropagation (not studied so far) and use of liquid medium.

SLS (the organized tissues) are the best source for the biosinthesis of crocin, picrocrocin, crocetin and safranal by tissue culture, however, continuous production of high quality SLS *in vitro* in sufficient quantity is a challenge for commercial upgradation of this technology. If the current status of research on this aspect is an indicative, there is a possibility of surpassing the contents of crocin, picrocrocin, crocetin and safranal in SLS compared to natural stigmas. Precursors improve crocin synthesis in SLS and a rare earth element, Ce^{3+} enhances crocin production in cell cultures (Otsuka *et al.* 1992; Zeng *et al.* 2003; Chen *et al.* 2004). Using the method of Loskutov *et al.* (1999) and combining it with incubation under continuous light (Zeng *et al.* 2003), precursors and rare earth elements might lead to higher concentration of metabolites in SLS. In comparison to SLS, cell culture system for production of crocin, crocetin, picrocrocin and safranal has the advantage of upscaling by use of bioreactors. Improvisation of the current cell culture protocols based on factors like media, casein hydrolysate, precursors, heavy metals/rare earth elements, temperature, light and a two-stage culture system, all of which improve either cell biomass or crocin biosynthesis, is required to develop bioreactor-based system for commercial production of important saffron metabolites in cell cultures. The crocin, crocetin, picrocrocin and safranal in saffron stigmas are synthesized via the terpenoid pathway. Studies on gene expression in saffron (Alvarez-Ortí *et al*. 2004; Castillo *et*

Table 2 Summary of *in vitro* research in *Crocus sativus*.

Explants used	Results	References
Callus/Somatic embryogenesis		
Corm	Callus, shoots	Ding *et al.* 1979
Corm	Callus, shoots	Ilahi *et al*. 1987
Corm	Callus	Isa *et al.* 1990
Apical buds	Callus, somatic embryogenesis	George *et al*. 1992
Corm	Somatic embryogenesis	Ahuja *et al.* 1994
Ovary	Callus, shoots	Igarashi *et al.* 1994
Corm, shoot, inflorescence, anthers	Callus, somatic embryogenesis	Milyaeva *et al.* 1995
Ovary	Callus	Castellar and Iborra 1997
Apical bud[a]	Callus, somatic embryogenesis	Piqueras *et al*. 1999
Shoots, corm	Somatic embryogenesis	Blázquez *et al*. 2004a, 2004b
Callus	Somatic embryogenesis	Blázquez *et al*. 2004a, 2004b
Shoot meristem	Somatic embryogenesis	Karamian 2004
Buds	Callus, shoots	Sharma *et al.* 2005
Apical bud	Callus, protoplasts, somatic embryogenesis	Darvishi *et al.* 2007
Corm, shoots, inflorescence, ovary, flower, stigma	Shoots, somatic embryogenesis	Karaoglu *et al.* 2007
Leaf	Callus, somatic embryogenesis, shoots	Raja *et al.* 2007
Corm	Callus, somatic embryogenesis, shoots, roots	Sheibani *et al.* 2007
Apical bud	Callus	Blázquez *et al*. 2009
Protoplast culture		
Calli from corms	Protoplasts, shoots	Isa *et al.* 1990
Direct shoot regeneration/ Microcrom formation		
Corm	Shoots	Homes *et al.* 1987
Apical bud	Shoots, microcorm	Plessner *et al*. 1990
In vitro shoots	Microcorm	Aguero and Tizio 1994
Corm, shoots	Shoots, microcorm, roots	Milyaeva *et al.* 1995
Ovary	Shoots, microcorm	Bhagyalakshmi 1999
Somatic embryos	Microcorm	Piqueras *et al*. 1999
Somatic embryos	Microcorm	Raja *et al.* 2007
Shoots	Shoots	Majourhat *et al.* 2007
Somatic embryos	Microcorm	Karaoglu *et al.* 2007
Somatic embryos	Microcorm	Sheibani *et al.* 2007
Corm, apical bud	Shoots, microcorm	Sharma *et al.* 2008
Apical bud	Shoots, plantlets	Sharifi *et al*. 2010
Stigma-like structure formation		
Ovary, stigma	SLS[b]	Sano and Himeno 1987
Ovary, stigma	SLS	Himeno and Sano 1987
Ovary	Parthenocarpic fruit	Chichiricco and Grilli Caiola 1987
Flower bud	SLS	Koyama *et al*. 1988
Anthers, petal, stigma, half ovary	SLS	Fakhrai and Evans 1990
Anthers, ovary	SLS	Sarma *et al.* 1990
Anthers, ovary	SLS	Sarma *et al.* 1991
Corolla, pistil	SLS	Ostuka *et al.* 1992
Stigma, style, ovary, ovule, petal	SLS	Kohda *et al*. 1993
Ovary	SLS	Castellar and Iborra 1997
Half ovary	SLS	Loskutov *et al*. 1999
Style, perianth	SLS	Ebrahimzadeh *et al*. 2000
Stamen	SLS	Zhao *et al.* 2001
Petal	Petal-like structures, SLS	Wang *et al*. 2002
Petal, stigma, style	SLS	Zeng *et al.* 2003
Style	Flower	Jun *et al*. 2007
Metabolite synthesis in cell cultures		
Pistil	Callus	Hori *et al.* 1988
Buds	Callus	Dufresne *et al.* 1997
Petal	Callus	Zeng *et al.* 2003[c]
Corm	Callus	Chen *et al.* 2003[c]
Corm	Callus	Chen *et al.* 2004[c]

[a]Also termed apical meristem and consists an apical bud along with about 2 mm or larger sided cube of corm tissue.
[b]SLS, stigma-like structures
[c]Only crocin production studied

al. 2005) are in infancy and none of the genes involved in biosynthesis of crocin, picrocrocin, crocetin and safranal has been isolated. The knowledge of these genes in the long run may open up the possibilities of improving yield of these metabolites in transgenic cells or plants.

Scale up of saffron tissue culture technologies for commercial exploitation in near future seems to be remote unless research efforts are intensified. Increasing industrial demand for crocetin esters, limited area under this crop and reduction in average yields during the recent years is expected strengthen research on production of crocin and other important metabolites of saffron stigmas in cell cultures or in SLS so that dependence on field grown saffron is minimized. In addition to this, there is a need to exploit tissue culture techniques to generate somaclonal variants so that genetic base of this genetically uniform crop (Agayev *et al.* 2009) may be widened and better yielding clones are generated.

ACKNOWLEDGEMENTS

KDS thanks the Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi for grant to work on saffron. AP thanks the Instituto de Desarrollo Regional (Universidad de Castilla La Mancha) at Albacete, Spain and the CEBAS (CSIC) at Murcia, Spain for supporting his research on saffron.

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How to reference: Author name(s) (2010) Title of chapter. In: Husaini AM (Ed) Saffron. Global Science Books, UK, pp. X-XX

Genomics and Transcriptomics of Saffron: New Tools to Unravel the Secrets of an Attractive Spice

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ABSTRACT

Saffron (*Crocus sativus* L.) is a triploid, sterile plant belonging to the *Iridaceae* family and it has been used as a spice and medicinal plant in the Mediterranean area for thousands of years. Saffron is currently considered the most expensive spice available on the global market. Nowadays, an in-depth knowledge of the genomic and transcriptomic organization of saffron represents the main step to fully elucidate the origins of *C. sativus* and the genetic basis of its organoleptic properties. A combination of EST sequencing, characterization of genetic polymorphisms, and "*omics* "approaches will be discussed as effective tools in saffron investigation.

Keywords: apocarotenoids, CCD, *Crocus sativus*

CONTENTS

INTRODUCTION

Saffron is the name given to the desiccated stigmas of *Crocus sativus* L., a triploid, sterile plant, propagated by corms, belonging to the family *Iridaceae* (Deo 2003). The word "saffron" comes from the Arabic: "za-faran" (yellow) and the powder obtained from the dried stigmas is renowned today as the world's most expensive spice.

Main properties of saffron are its bright orange-red colour, the bitter taste, flavour and aromas that confer to many traditional and seafood meals, especially in Asian and western European countries. In ancient times, frescoes paintings from Knossos, Crete, (dated back to 1700 B.C.) witness the use of saffron in the Minoan civilization (Deo 2003). In fact, *C. sativus* was probably domesticated by Minoan farmers between 3000 and 1600 B.C., when *Crocus* plants were selected on the basis of their pigmented stigmas. Saffron was also consumed by Romans and Greeks, who believed in its aphrodisiac properties and was used both as spice for food and wines, as a dye and in the preparation of several perfumes (Hill 2004). From the Middle Ages to the industrial revolution, the diffusion of saffron was accompanied by a constant increase in its commercial value. Once perceived as a "luxury" spice, saffron began to be adulterated with low-quality colouring agents and inorganic ingredients. In recent years, a novel interest has emerged with respect to the pharmacological properties and antioxidant potential derived from the consumption of saffron in human diet.

The red stigmas of *Crocus* accumulate three different apocarotenoids (i.e. products derived by the enzymatic cleavage of a carotenoid precursor): crocin, picrocrocin, and safranal (**Fig. 1**), which are responsible, respectively, for the colour, taste and aroma of saffron (Kanakis *et al.* 2004). The ability to synthesize these compounds is not common across species: picrocrocin and crocin, in fact, have only been identified in stigma tissues of some *Crocus* species and few others species such as *Buddleja* (Liao *et al.* 1999) and *Gardenia* (Pfister *et al.* 1996). Because of the predominant accumulation of apocarotenoids in the stigmas of *C. sativus*, and due to their potential antioxidant effects, their biosynthesis has been extensively investigated.

BOTANY OF *CROCUS SATIVUS*

C. sativus shows perennial, herbaceous, rosette growth and has permanent underground stem bases, called bulb or corm, almost spherical with a 3-5 cm diameter (Hill 2004; Molina *et al.* 2004). *C. sativus* is an autumn flowering geophyte with subhysteranthous behaviour and leaf emergence coincides or occurs shortly after flowering, withering at the onset of the dry season, while during late spring and most of summer plants show no aboveground organs or roots (condition usually called "dormancy"). Flower initiation usually occurs during this period and its formation is usually restricted to the apical and dominant bud in non-flowering shoot-derived corms, while it can occur in two or three apical buds in flowering shoot-derived corms (Molina *et al.* 2004). Saffron growth cycle usually lasts around 220 days and favourable climatic conditions for high yields of

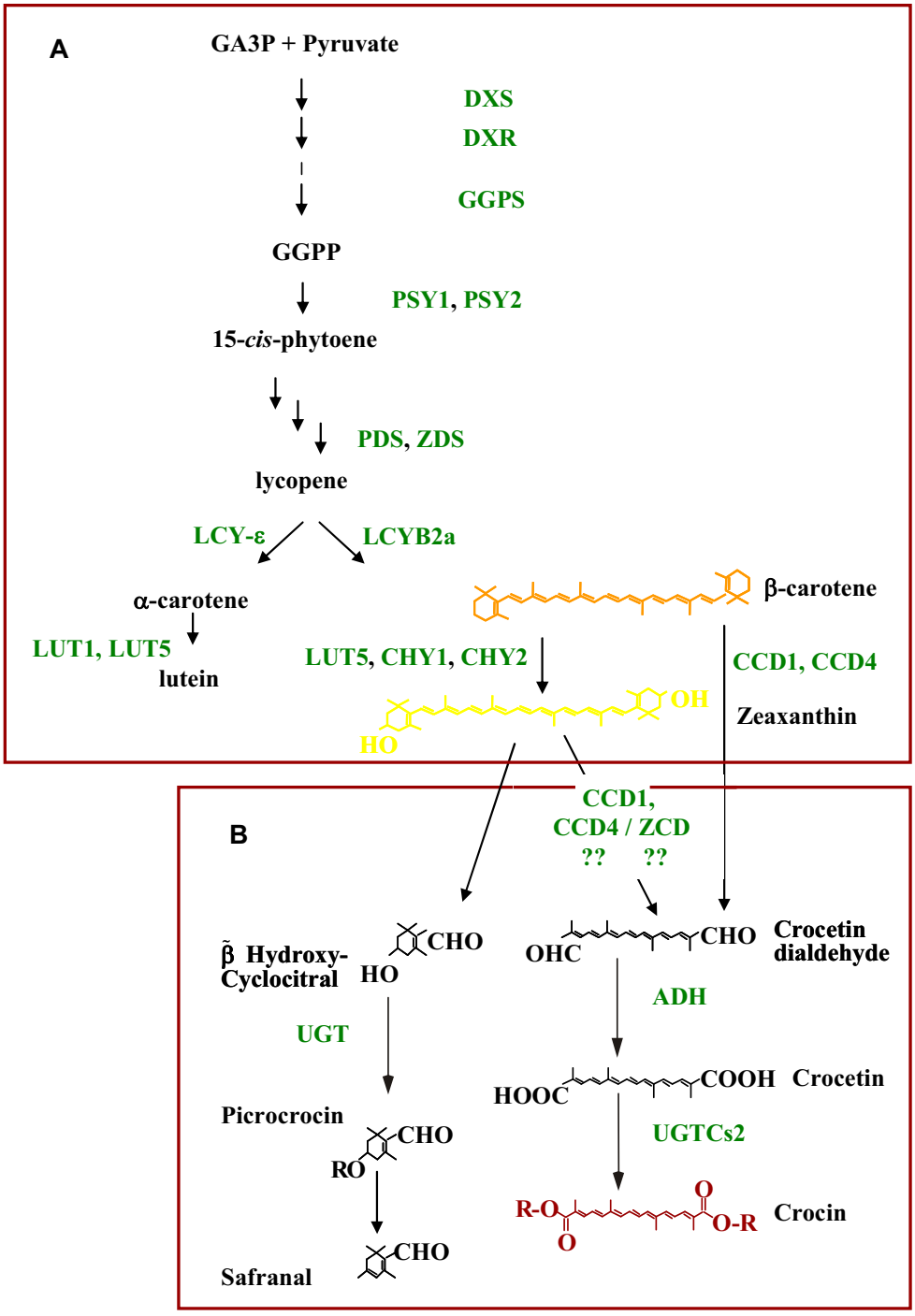

Fig. 1 Biosynthetic pathway of three major carotenoid derivatives, crocetin, picrocrocin, and safranal in *C. sativus* stigma. (**A**) Carotenoid biosynthetic pathway until zeaxanthin (Diretto *et al.* 2007). GA3P, glyceraldehide 3-phosphate; DXS, 1-deoxyxylulose 5-phosphate (DOXP) syntase; DXR, DOXP reductoisomerase; GGPP, geranyl geranyl diphosphate; GGPS, geranyl geranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CHY, β-carotene hydroxylase; LUT1, ε-carotene hydroxylase; LUT5, ε–β carotene hydroxylase; LCY-ε, lycopene ε-cyclase LCYB2a, lycopene β cyclase isolated in *C. sativus* (Ahrazem *et al.* 2009); (**B**) Biosynthetic pathway of saffron apocarotenoids (according to Bouvier *et al*. 2003; Moraga *et al*. 2008). CCD, carotenoid cleavage dioxygenase; ADH, Aldehyde dehydrogenase; UGT, UDPG-Glucosyltransferases; UGTCs2, UDPG-Glucosyltransferases isolated in *C. sativus* (Moraga *et al*. 2004); ZCD, zeaxanthin cleavage dioxygenase (Bouvier *et al.* 2003).

saffron are rainfall in the autumn, warm summers and mild winters. Water requirements are low and garden (clay sand) soils allow the optimum growth. These features, together with the very low harvest make saffron a highly remarkable agrological and eco-physiological species.

GEOGRAPHICAL DISTRIBUTION OF *CROCUS SATIVUS* AND SAFFRON PROPERTIES

Saffron is considered the highest priced spice in the world (on average, 500 $/kg) (Hill 2004). Its high price is due to the direct manual labour required for its cultivation, harvesting and handling. One stigma of saffron weighs about 2 mg, each flower has three stigmata, and 150,000 flowers must be carefully picked in order to produce 1 kg of spice.

Virtually, saffron is produced in a wide geographical belt extending from the Mediterranean area in the west to the Kashmir region in the east. All the continents outside of this zone, except for Antarctica, produce smaller amounts. Annual worldwide production amounts to around 205 tonnes (Schmidt *et al.* 2007), including whole threads and powder. Iran, Spain, India, Greece, Azerbaijan, Morocco, and Italy (in decreasing order of production) dominate the world saffron market, with Iran and Spain accounting for

80% of the total. Despite numerous cultivation efforts in countries especially in Austria, United Kingdom, Germany, and Switzerland, only few locales cultivate saffron in Northern and Central Europe.

Saffron has a sweetish aromatic odour and a bitter taste. It is mainly used as spice or condiment, adding its faint, delicate aroma, pleasing flavour and magnificent yellow colour to enhance palatability. The range of foods that have been spiced with saffron is wide, including cream or cottage cheese, chicken and meat, rice, cakes, mayonnaise, mustard, chocolate and liqueurs (Basker *et al.* 1983). The commercial quality of saffron depends heavily on its colouring strength, bitterness and aroma intensity. Saffron is also considered a highly valued medicinal plant and many pharmaceutical uses have been reported so far (Schmidt *et al.* 2007). Traditionally, it has been used against cramps, bronchospasms, liver and menstruation disorders (Abdullaev 2003). Very important applications are also in supportive treatments of various forms of cancers (Dhar *et al*. 2009), in anti-inflammatory responses (Hosseinzadeh *et al*. 2002) and in anti-depressive therapies (Hosseinzadeh *et al*. 2004).

APOCAROTENOID BIOSYNTHESIS AND MOLECULAR INVESTIGATION IN SAFFRON

Plant apocarotenoid biosynthesis starts with geranyl diphosphate that is deemed to be the universal precursor for monoterpenes, key constituents of flower, fruit, and spice plant aromas (Croteau *et al.* 2000). Geranyl diphosphate is converted into geraniol by the activity of geraniol synthase (direct pathway). In several plants, geraniol is readily oxidized to geranial by alcohol dehydrogenases but, anyway, it has also been shown that geranial and other apocarotenoids can be formed *in vitro* by oxidative cleavage of lycopene.

The synthesis can also proceed indirectly, through the oxidative cleavage of carotenoids: farnesyl acetone and geranyl acetone are produced from phytoene; pseudoionone, neral and geranial are instead obtained from the cleavage of neurosporene, pro-lycopene and lycopene; finally, β-carotene is the precursor of a third group of volatiles including β-cyclocitral, β-ionone and dihydroactinodiolide. Citral, a mixture of *cis* and *trans* noncyclic monoterpene aldehyde isomers (neral and geranial, respectively) possesses an agreeable scent, reminiscent of lemon (Lewinsohn *et al.* 2005) and it is a major component of lemon basil (*Ocimum basilicum* L., Lamiaceae), lemongrass (*Cymbopogon citratus*, Poaceae), litsea (*Litsea cubeba* Pers., Lauraceae), and other lemon-scented aromatic plants. Citral has also a major impact in the aroma of tomato and watermelon (*Citrullus lanatus*), two fruits accumulating high levels of the tetraterpene red pigment lycopene (Lewinsohn *et al.* 2005).

It is presently unknown whether citral accumulating in lycopene-rich fruits is directly derived from geranyl diphosphate or if it is produced by oxidative degradation of lycopene. Wild-type tomatoes also accumulate specific non-cyclic volatile norisoprenoids, such as 6-methyl-5-hepten-2-one, farnesyl acetone, (*E*,*E*)-pseudoionone, 2,3-epoxy-geranial, 2,6-dimethylhept-5-1-al, geranyl acetone, and dihydro-*apo*-farnesal, as well as the monoterpene aldehydes geranial and neral (citral) and the cyclic norisoprenoid α-ionone (Lewinsohn *et al.* 2005).

Crocus flowers show red style branches, which, upon desiccation and once reduced to a powder, constitute the spice saffron. It has been proposed that the biogenesis of the three major carotenoid derivatives, crocetin glycosides (crocins), picrocrocin, and safranal, which are responsible for saffron colour, bitter taste and aroma, is derived from the oxidative cleavage of the carotenoid zeaxanthin (Bouvier *et al.* 2003) (**Fig. 1**). This step leads to the formation of a polyene molecule (crocetin dialdehyde) and two identical β-ionone molecules, hydroxyl-β-cyclocytral. The oxidated form of crocetin dialdehyde (crocetin) constitutes the substrate of another reaction probably catalyzed by an UDP-glucosyltransferase, with the formation of glucosyl esters of crocetin (named crocins, which can be further classified in different subtypes on the basis of the transferred sugar moiety). On the other side of the pathway, the β-ionone hydroxyl-β-cyclocytral, derived from the initial cleavage of zeaxanthin, is thought to be converted by an UDP-glucosyltransferase to picrocrocin and safranal (Moraga *et al.* 2004). The first genes that were cloned, identified and functionally characterized in *Crocus* are *CsZCD* (zeaxanthin cleavage dioxygenase) and *CsCCD* (carotenoid (9', 10')-cleavage dioxygenase) (Bouvier *et al.* 2003). The expression of *CsZCD* seems to be restricted to the style branch tissues and it is enhanced under dehydration stress, whereas *CsCCD* is expressed in a constitutive manner in flower and leaf tissues and irrespectively of dehydration stress. Bouvier and co-authors (Bouvier *et al.* 2003) suggested the existence of a stepwise sequence involving the oxidative cleavage of zeaxanthin inside the chromoplasts followed by the sequestration of modified water-soluble derivatives into the central vacuole.

The subsequent step involves glucosylation of crocetin and β-hydroxy-ciclocytral, with the formation of, respectively, crocin and picrocrocin. These glucosylation reactions are catalysed by various glucosyltransferases (GTases), belonging to a class of enzymes involved in the biosynthesis of several plant secondary metabolites (glycoalkaloids, anthocyanins, betalains, etc.). The Gómez-Gómez group cloned and studied the expression of a gene coding for a saffron glucosyltransferase able to glucosylate crocetin (Moraga *et al.* 2004). Glucosylation of crocetin is very important since it confers stability and water solubility to the pigment and improves its bioavailability and, thus, its pharmaceutical interest. Additional genes involved in saffron carotenoid biosynthesis have been characterized so far: partial clones encoding phytoene desaturase, phytoene synthase and carotenoid dioxygenases from stigma tissues have been isolated, and their expression has been analyzed during stigma development and carotenoid accumulation. High expression level of carotenoid dioxygenase 3 clone (*CsCCD3*) suggests that the gene product could be involved in saffron apocarotenoid biosynthesis (Rubio *et al.* 2004).

Castillo and co-workers observed that expression level of *CsBCH* (β-ring hydroxylase) is dependent from the relative levels of zeaxanthin in the stigma, suggesting that activity of this enzyme could represent a limiting step in the apocarotenoid formation (Castillo *et al.* 2005).

More recently, four additional genes encoding carotenoid cleavage dioxygenase have been isolated from *C. sativus*: *CsCCD1a*, *CsCCD1b*, *CsCCD4a* and *CsCCD4b* which show a very variable pattern of expression among different tissues. *CsCCD1b* was expressed only in stigma tissues and the expression levels of both *CsCCD4a-4b* correlated with the accumulation of β-ionone during stigma development (Rubio *et al.* 2008). Moreover, bioinformatic analyses have showed that the deduced amino acid sequences of several carotenoid dioxygenases from a variety of plant organisms cluster into four distinct subfamilies: CCD1, CCD4, NCED and a fourth class including both CCD7 and CCD8. It has been shown that CCD1 family possesses cleavage activity on a variety of carotenoid substrates, while members of CCD4 family would only be able to cleave β-carotene. Rubio and co-workers also assessed that, in their experimental conditions, CsZCD enzyme lacks of cleavage activity; expression of *CsZCD* gene in zeaxanthin-accumulating *E. coli* strain resulted in, unexpectedly, no cleavage activity. CsZCD enzyme would, thus, represent a truncated N-terminal form with respect to the CCD4 protein, lacking of plastid target sequence. On the basis of the discordant data between the two groups (Rubio and co-workers, Bouvier and co-workers), CsZCD enzyme characterization and its catalytic activity would need a more detailed and accurate re-evaluation.

Recently, studies of a number of saffron enzymes involved in flavonoid glucosylation (flavonoid glucosyltransferares, Rubio-Moraga *et al.* 2009) and carotenoid biosynthesis (lycopene β-cyclase, Ahrazem *et al.* 2009) have been

performed, increasing the number of candidate genes which are responsible for the high-valuable saffron organoleptic features.

GENOMIC ORGANIZATION IN *CROCUS SATIVUS*

A large genome size (around 30,000 Mbp and, thus, twice, 60 and 240 times larger than, respectively, *Triticum estivum*, *Oriza sativa* and *Arabidopsis thaliana*) has been estimated for *C. sativus* on the basis of the size of the diploid specie *C. vernus* (11,000 Mbp; Chichiriccò 1984).

Saffron is a triploid specie with basic chromosome number of x=8 (Chichiriccò 1984). The karyotype of *C. sativus* has been studied by several authors and on different ecotypes from several countries (Azerbaijan, Iran, Italy, Turkey, France and United Kingdom) and it is always resulted as 2n=3x=24, without any significant karyologycal difference. The accepted karyotype is, then, composed of 8 triplets: subacrocentric (1, 2), metacentric (3, 4 and 8) and submetacentric (6 and 7) chromosomes. Three chromosomes in each triplet, as a rule, are similar although in some triplets one of them is infrequently distinguishable from the others. Triplet 5 shows a marked as it contains two distinct chromosomes subtypes: chromosome 5(1), metacentric, and chromosome 5(2,3), subacrocentric and smaller.

Using a combination of genetic, molecular and cytological methods, genomes of several *Crocus* species have been investigated: Heslop-Harrison and co-workers (Frello *et al.* 2000), for example, through a recombinant DNA library, isolated eight clones of repetitive DNA in *C. vernus* Hill. The DNA organization was analyzed by *in situ* hybridization and Southern analyses in a broad range of *Crocus* species. Sequence analysis evidenced that all 8 clones were non homologous, and suggesting, thus, the presence of 8 different sequence-families. Almost all clones, analyzed by *in situ* hybridization, displayed a dispersed organization at high copy numbers on all chromosomes of the *C. vernus* genome. In only one case, a specific sequence has been found showing high homology to the reverse transcriptase gene of Ty1-*copia*-like retrotransposons. The genomic distribution of clones seemed to be discordant with the taxonomy classification, therefore this data suggested that a more detailed analysis of phylogeny and taxonomic structure should be done. Moreover, the genomic distribution and organization of two clones of highly repetitive DNA previously described (Frello *et al.* 2000), were further studied (Frello *et al.* 2004). The sequences of the two clones were 85% identical; the presence of these sequences was monitored in 54 *Crocus* taxa and in some species of other genera. These findings suggested that both sequences were specific to *Crocus*. Unfortunately, the distribution of hybridization signal across the genus showed poor agreement with the taxonomic structure, confirming that taxonomy of the *Crocus* genus might need additional and novel re-evaluation.

More recently, many efforts have been performed to achieve a better understanding of the genomic organization of *Crocus* species. Seberg *et al.* (2009) presented the analysis of a proposed barcode set of genes (Chase *et al.* 2007) in the genus *Crocus*. *RpoC1*, *matK* and *tmH-psbA* regions were analyzed on 86 species of the *Crocus* genus and the proposing sets were further extended with several other genomic regions to obtain a final diagnostic set for 79 out of the 86 analyzed species. The authors asserted that, although barcoding is still unable to identify more than 75% of the known species, it represents a very promising and powerful system and more efforts in this technology are thus envisaged in the near future.

In a more recent paper, Moraga *et al.* (2009) analyzed the RAPD profile of 43 isolates of *C. sativus* to determine the morphism of this species. Using three different approaches (random amplified polimorphic DNA, intersimple sequence repeats (ISSR) and microsatellite analysis), they assessed the variability of saffron from several different geographic areas and concluding that *C. sativus* is a monomorphic species.

A new genome walking approach named "rolling circle amplification of genomic templates for inverse PCR" (RCA-GIP) has been proposed (Tsaftaris *et al.* 2009). This method consists of a rolling circle amplification of the circular DNA generated from restriction analysis of genomic DNA by using different enzymes and through a subsequent ligation with appropriate adaptors. Using this method, four promoter regions of flowering genes (Tsaftaris *et al*. 2007) from *C. sativus* were isolated. The promoter analysis showed a presence of common promoter elements as the TATA box and binding motifs for transcriptional factors. The RCA-GIP method allowed the isolation of genomic sequence flanking several genes; this is an important step to understanding the complicated mechanisms of gene regulation.

TRANSCRIPTIONAL ANALYSIS OF *CROCUS SATIVUS*

The characterization of the saffron transcriptome is a very important starting point to shed light on several high-valuable biological processes such as the molecular basis of flavour and colour biogenesis and the genomic organization of *Iridaceae*. The group of the authors presenting this review carried out a large-scale study dealing with the construction of an Expressed Sequence Tags (EST) database from saffron stigmas. In this work (D'Agostino *et al.* 2007), espression profiling of mature stigmas was evaluated by means of collection and sequencing of an EST pool. Bioinformatic analysis of the entire dataset provided a first general overview of the transcriptome organization of this species with a particular emphasis on the organ involved in saffron spice production.

The global sequencing of a cDNA library produced from mature stigmas of *C. sativus* coupled to extensive and bioinformatic analyses of sequence data allowed the construction of the first saffron database (www.saffrongenes.org). "Saffrongenes" is a freely available resource for the research community involved in saffron genomics (D'Agostino *et al.* 2007).

Several bioinformatic analyses were performed; 7965 ESTs were obtained through remotion of the vector regions and were subsequently subjected to a batch analysis (BlastX) in order to identify significant homologies. Within the pool of EST selected (7965), 5355 EST (67%) give no significant homologies and 2610 (33%) ESTs showed significant homologies. The 2610 ESTs were further separated in non-redundant (the typologies of homology, NR-set) and redundant (EST belonging to each classes, R-set) sets. NR-set (392 elements) accounts the independent classes of homology excluding their occurrence (number of EST belonging to a specific class), whereas redundant R-set includes the homology classes and the number of EST in each classes (2610 elements). A manual classification in 10 functional classes was performed (**Fig. 2**) for both datasets in order to identify most representative classes: Regulation (19% NR-set, 16% R-set), metabolism (19% NR-set, 17% R-set), stress response (16% NR-set, 19% R-set).

A further classification and more accurate investigation were conducted on the EST initial pool using a dedicated platform of bioinformatic clustering, assembly and annotation of EST, named ParPEST (D'Agostino *et al.* 2005). The analyzed pool comprises 6,603 high quality ESTs from a saffron mature stigma cDNA library. The ESTs have been grouped into 1,893 clusters, each corresponding to a different expressed gene, and subjected to a subsequent annotation. Evaluation of homologies after BlastX analysis evidenced the high expression level of some transcript contigs (TCs). In the context of ESTs analysis, the number of ESTs members in each unique gene is an indication of the expression level of that gene. The most represented homologies in *C. sativus* stigmas were grouped in three different ontology classes: molecular function, biological process and cellular component. Catalytic activity and transport were the most represented category in, res-

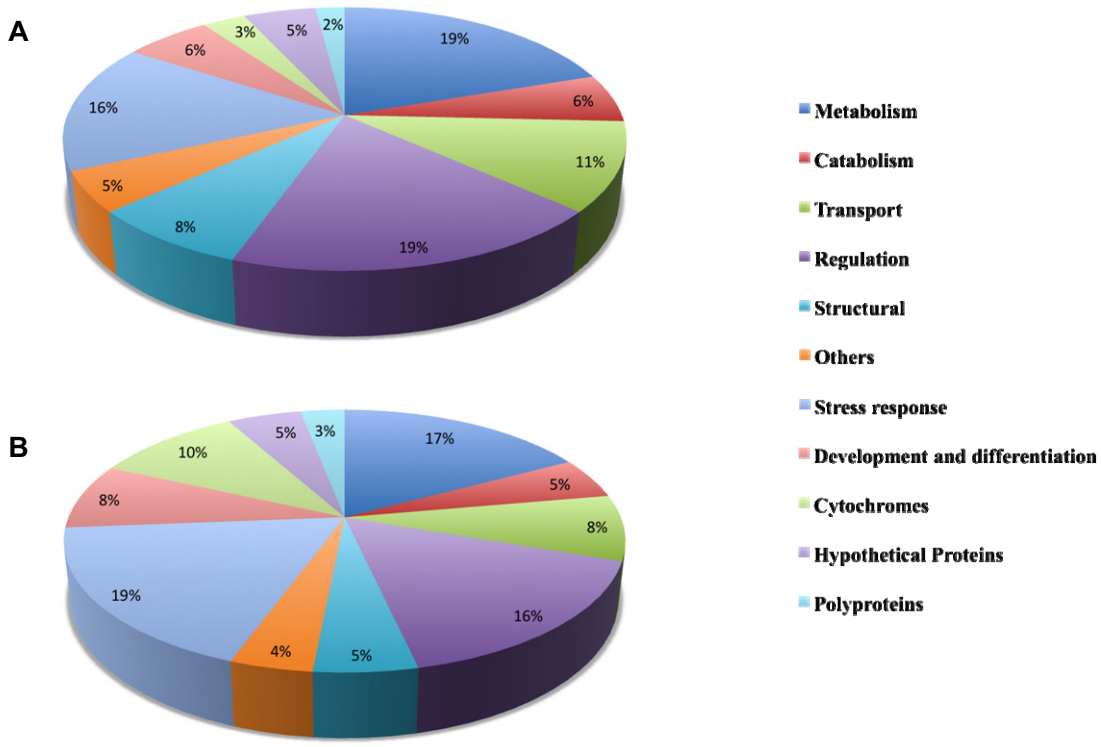

Fig. 2 Functional classification of ESTs: (**A**) non redundant set, (**B**) redundant set.

pectively, molecular function and biological process classes, whereas a more uniform distribution was observed in the cellular component class, with similar levels among the three cellular compartments: plastid, mitochondrion and cytoplasmic membranes. The most highly expressed TC, Cl000057:2 (547 ESTs), revealed homology to short chain dehydrogenases and is homologue to *TASSELSEED2 (TS2)* of maize that is involved in sex organ differentiation and in the genetically determination of programmed pistill cell death (Calderon-Urrea *et al.* 1999). Moreover, many P450 (122 ESTs) and b5 (45 ESTs) cytochromes were recovered from the sequencing of saffron stigmas libraries; the high abundance of these transcripts may be related to the primary role that cytochromes have in stigma metabolism and development. Several ESTs clustered into TCs related to enzymatic activities which are specifically involved in secondary metabolisms (carboxyl methyltransferase (42 ESTs) and UDP-gycosyltransferase (33 ESTs)) while some other TCs, encoding putative proteins with high homology to heat shock proteins of *Arabidopsis thaliana* (TC, Cl001114:3, 104 ESTs), lipid transfer proteins (94 ESTs) and to transcription factors such as the Myb-like factors (54 ESTs) and MADS box (36 ESTs) were also identified. The Saffron-gene database (www.saffrongenes.org) has been designed to manage and to explore the EST collection from saffron stigmas, providing a reference for the expression pattern analysis in this tissue as well as a primary view of the genomic properties of this species, and the database represents the first reference collection for the genomics of *Iridaceae*, for the molecular biology of stigma biogenesis, as well as for the metabolic pathways underlying saffron secondary metabolism.

A "local" transcriptomic approach has been also performed to study the expression of specific classes of genes: Tsaftaris and collaborators provided, for example, a detailed investigation of regulatory genes involved in flowering time and flower development (Tsaftaris *et al.* 2009). In this study, a combination of conventional (5'- and 3'-RACE PCR) and new methods (Rolling Circle Amplification RACE, and familyRCA-RACE) has been employed to further characterize the structure and expression of specific gene families. Several full-length cDNA clones encoding MADS-box transcription factor proteins involved in flower formation were cloned and characterized; within this group, five PISTILLATA/GLOBOSA-like (PI/GLO-like) MADS-box genes were isolated and gene expression studies detected their expression, in the outer whorl tepals which are the sepals in most typical flowers (Kalivas *et al.* 2007).

In another study, the Gómez-Gómez group (Rubio Moraga *et al.* 2009) used a combination of approaches to study the accumulation of colour and aroma compounds during stigma development performing an *in silico* screening of the stigma cDNA database previously described (D'Agostino *et al.* 2007) and a GC-MS profiling of stigma metabolites, in order to identify candidate genes encoding enzymes involved in saffron volatile biosynthesis. In this way, authors identified several genes showing highly increased expression during stigma development: this group comprises two putative terpene synthases, TS1 and TS2 and two carotenoid gene transcripts, *CsPSY* (phytoene synthase) and *CsPDS* (phytoene desaturase). Taken together, these data suggest expression pattern of several candidate genes is strongly correlated with volatile production and organoleptic characteristics during stigma development.

In any case, a detailed large-scale analysis of the transcriptome and metabolome of *Crocus species* should provide additional details on early developmental stages of saffron stigmas, where the transcription of genes involved in flavour and aroma production leads to the accumulation of secondary metabolites.

CONCLUSIONS

In recent years, a combination of different approaches has been employed in *Crocus* species to better characterize the available genetic resources, as well as the organization and composition of its genome and the transcriptional activity of saffron-accumulating tissues. Several genes responsible for flavour, aroma production, carotenoid and flavonoid accumulation have been isolated and functionally characterized. However, the metabolism of the stigmas of *Crocus* is still poorly characterized, despite the recent progresses made in elucidating the biosynthesis and accumulation of the major apocarotenoids. A more extensive investigation of *Crocus* metabolites involved in primary/secondary metabolism, which accumulate during stigma development, is thus

needed, with a particular emphasis on those volatile compounds which are presumably involved in the generation of the saffron aroma. In the near future, the rapid evolution of next-generation DNA sequencing technologies and the recent progresses at the level of sample throughput and instrumental resolution of analytical platforms for metabolite structural characterization (e.g., high-resolution mass spectrometers) will offer a promising, combined approach to correlate the genetic determinants with the corresponding biochemical phenotypes. The detailed elucidation of the genetic and biochemical basis responsible for saffron organoleptic qualities and pharmaceutical properties will thus require an integration of different high-throughput "*-omics"* techniques.

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How to reference: Author name(s) (2010) Title of chapter. In: Husaini AM (Ed) Saffron. Global Science Books, UK, pp. X-XX

Understanding Saffron Biology using Bioinformatics Tools

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ABSTRACT

Saffron (*Crocus sativus* L.) is a sterile triploid plant that belongs to the Iridaceae (Liliales, monocots). It is used as a spice and also has diverse medicinal properties. Its genome is of relatively large size and is poorly characterized. There is a need to integrate various approaches like transcriptomics, proteomics and metabolomics in order to shed light on and to dissect the molecular basis of flavour and color biogenesis, genomic organization and biology of the gynoecium of saffron. However, the biological data generated from such biotechnological advances needs parallel evolution of bioinformatics tools for data analyses, integration, modelling and prediction. Bioinformatics can play an enormous technical role in the sequence-level structural characterization of saffron genomic DNA. Such tools can also help in appreciating the extent of diversity of various geographic or genetic groups of cultivated saffron to infer relationships between groups and accessions. The information derived can be utilized for constructing biological pathways involved in the biosynthesis of principal components of saffron.

Keywords: *Crocus sativus*, genomics, *in silico*, metabolomics, proteomics, transcriptomics

CONTENTS

INTRODUCTION

Saffron (*Crocus sativus* L.) is a sterile triploid plant that is naturally propagated vegetatively by daughter corms developing on a mother corm. It is a member of the Iridaceae (Liliales, Monocots) whose genomes are relatively large and are poorly characterized (Fernández 2004). Among the 85 species belonging to the genus *Crocus*, saffron is the most fascinating and intriguing species. The word "saffron" is derived from the Arabic word "zafran", which translates to "yellow". Saffron was an important cultivated plant during the period of the Ottoman Empire, but its production has decreased with time (Arslan *et al*. 2007). Total world saffron production is estimated at 220,000 kg (220 metric tones), of which about 90% is produced in Khorasan Province, Iran (Jahan and Jahani 2007) and the remaining in Greece, Spain, Italy and India (Kashmir). Saffron introduction into new areas should be encouraged as it is a unique crop in terms of its potential and is recognized as red gold (Yadollahi *et al.* 2007). It is the highest priced spice in the world at around \$500 kg^{-1} of saffron (Fernández 2007).

PRINCIPAL BIOACTIVE MOLECULES

Saffron, the dried red stigmas of *C. sativus*, is used as flavouring and colouring agent. Owing to extremely high demand from the dye, perfumery and flavouring industries, it is one of the most expensive spices on earth. A complex mixture of volatile and non-volatile compounds contributes to the overall aroma and flavour of saffron (Tarantilis and Polissiou 1997). For colour, the principal pigment is crocin, for smell the main component is safranal and for the special bitter flavour the main compound is the glycoside picrocrocin (Basker 1999). These compounds are derived from oxidative cleavage of the carotenoid zeaxanthin (Bouvier *et al*. 2003; Moraga *et al.* 2004). Recent studies have revealed a different volatile composition in unprocessed stigma tissues (Rubio *et al.* 2008, 2009). Recently bioinformatics tools for sequence homology allowed the identification and characterization of orthologs of carotenoid cleavage dioxygenase (CCD) involved in production of aroma from *C. sativus* (Bouvier *et al.* 2003; Rubio *et al.* 2008).

Saffron stigmas and corms are characterized by the presence of antifungal saponins (Hosseinzadeh and Younesi 2002) and a large number of defence proteins capable of binding to chitin and chitin oligosaccharides. In fact, a new class of defense chitinase namely Safchi A has recently been isolated from saffron (Castillo *et al.* 2007; Castillo and Gómez-Gómez 2009). Furthermore, different phenolic compounds (pyrogallic acid, kaempferol, *p*-coumaric acid and gallic acid) involved in stress responses have also been identified (Crungoo *et al*. 1986; Ebrahimzadeh *et al.* 1997). Peroxidase, catalase and superoxide dismutase activities have been detected in saffron corms in different develop-

mental stages (Keyhani and Keyhani 2004; Keyhani *et al.* 2006), and genomic approaches have enabled the identification of partial sequence homologues for these enzymes. Bioinformatics analysis of corm and stigma libraries from Crocus has identified several genes associated with defence responses (D'Agostino *et al.* 2007).

Apart from its use as a spice, saffron has been used for medicinal purposes as well (Schmidt *et al.* 2007). The evidence indicates that saffron possesses anticancer activity against a wide spectrum of tumors, such as leukemia, ovarian carcinoma, colon adenocarcinoma, rhabdomyosarcoma, papilloma, squamous cell carcinoma, and soft tissue sarcoma. In addition, saffron can be used to cure coronary heart disease and hepatitis, and to promote immunity. Most of these medicinal properties of saffron are due to crocin, safranal, picrocrocin and β-carotene (Abdullaev 2003; Hosseinzadeh *et al*. 2002; Abdullaev and Espinosa-Aguirre 2004; Hosseinzadeh *et al*. 2004; Das *et al*. 2009; Dhar *et al.* 2009).

All these attributes make saffron an important crop for genome prospecting and demand approaches to build upon the knowledgebase of saffron in terms of genes, proteins and metabolites so as to provide researchers a platform for understanding the biogenesis of biologically important saffron metabolites. This would help to remodel or engineer such biosynthetic pathways to enhance the accumulation of important metabolites and to provide clues to break the jinx of complex disease mechanisms by saffron metabolites.

PRINCIPAL METABOLIC PATHWAYS AND GENES

Carotenogenesis in ripening fruit and petals has been studied extensively (Hugueney *et al.* 1996; Hirschberg 2001; Moehs *et al.* 2001; Zhu *et al.* 2002, 2003; Kato *et al.* 2004). In flowers and fruits high concentration of carotenoids is correlated with upregulation of genes that enhance the flux of the biosynthetic pathway (Botella-Pavía and Rodríguez-Concepción 2006). In these tissues, development and carotenoid accumulation occurs alongside chloroplast to chromoplast transition while peculiarly in *C. sativus*, stigma development and carotenoid accumulation occurs concomitantly with the amyloplast to chromoplast transition and the stigma never turns green during this process (Grilli-Caiola and Canini 2004; Castillo *et al.* 2005). These carotenoids serve as precursors of physiologically important apocarotenoids of which crocetin represents an important plant pigment of economic value (Walhberg and Eklund 1998). Crocetin is synthesised by *C. sativus* and other related species, *Jacquinia angustifolia* (Eugster *et al.* 1969), *Coleus forskolii* (Tandon *et al.* 1979), *Gardenia jasminoides* (Pfister *et al.* 1996) and *Buddleja* (Liao *et al.* 1999), but none of these species accumulate this metabolite at levels as high as those present in saffron.

Biosynthesis of apocarotenoids (crocetin, picrocrocin and saffranal) involves three interconnected processes: MEP pathway for the supply of precursors, carotenoid biosynthesis and subsequent cleavage of these carotenoids into apocarotenoids. MEP pathway provides geranyl geranyl diphosphate which acts as building block for the biosynthesis of phytoene. This step is catalysed by phytoene synthase and is the first rate limiting step in carotenoid biosynthetic pathway. Phytoene is converted into *trans*-lycopene by the action of four different enzymes viz phytoene desaturase (PDS), carotene desaturase (ZDS), zeta carotene isomerase (ZISO) and carotenoid isomerase (CRTISO). *Trans*-lycopene is further converted into beta carotene by lycopene beta cyclase which is subsequently converted into zeaxanthin by beta carotene cyclohydrolase. The zeaxanthin thus produced is converted into apocarotenoids by the action of zeaxanthin cleavage dioxygenase. Using bioinformatics approach like sequence homology many genes of these pathways have been isolated from *Crocus*. The first genes that were cloned, identified and functionally characterized in *Crocus* are *CsZCD* (zeaxanthin cleavage dioxygenase) and *CsCCD* (carotenoid (9',10')-cleavage dioxygenase (Bouvier *et al.* 2003). Bouvier *et al.* (2003) suggested the existence of a stepwise sequence involving the oxidative cleavage of zeaxanthin inside the chromoplasts followed by the sequestration of modified water-soluble derivatives into the central vacuole. The subsequent step involves glucosylation of crocetin and β-hydroxy-ciclocytral, with the formation of crocin and picrocrocin, respectively. These glucosylation reactions are catalysed by various glucosyltransferases (GTases) (Rubio *et al.* 2004). Glucosylation confers stability, water solubility and improves bioavailability of crocetin pigment. Additional genes involved in saffron carotenoid biosynthesis have been characterized and recently genes for phytoene synthase (PSY), lycopene cyclase (LYC), and carotene hydroxylase (CHY) have been identified and isolated from stigma tissue (Castillo *et al.* 2005; Ahrazem *et al.* 2009) which might play an important role in the high apocarotenoid accumulation in stigma tissue. High apocarotenoid biosynthesis in saffron has also been correlated with high expression of carotenoid dioxygenase (*CsCCD3*) gene (Rubio *et al.* 2004). More recently, four additional genes *CsCCD1a*, *CsCCD1b*, *CsCCD4a* and *CsCCD4b* encoding carotenoid cleavage dioxygenase have been isolated from Crocus (Rubio *et al.* 2008). Bioinformatic analyses have shown that the deduced amino acid sequences of several carotenoid dioxygenases from a variety of plant organisms cluster into four distinct subfamilies: CCD1, CCD4, NCED and a class including both CCD7 and CCD8.

In order to understand the aforementioned cellular metabolic systems in saffron, identifying the functions of enzymatic genes is no longer satisfactory; instead we need to decipher the coordination and interaction among various metabolic pathways. In this context, other high throughput experiments, including genomics, transcriptomics, proteomics, and metabolomics provide us with the complementary information to elucidate such coordination. Here bioinformatics is needed for biological sequence analyses, transcriptome analyses, computational proteomics, computational metabolomics, bio-ontologies and biological databases. Thus bioinformatics can play a great role for data integration and analyses, and make sense out of different experimental data (**Table 1**).

GENOMICS AND TRANSCRIPTOMICS

Saffron is a triploid with karyotype 2n=3x=24 comprising of 8 triplets: triplets 1 and 2 include subacrocentric, triplets 3, 4 and 8 metacentric, triplets 6 and 7 submetacentric chromosomes and triplet 5 which shows an extreme difference by containing two kinds of chromosomes: chromosome 5(1), metacentric, and chromosomes 5(2,3), subacrocentric and smaller. Genomes of several *Crocus* species have been investigated using a combination of genetic, molecular and cytological methods (Frello *et al.* 2000, 2004). More recently, many efforts have been made for a better understanding of the genomic organization of *Crocus* species. Seberg *et al.* (2009) presented the analysis of a proposed barcode sets (Chase *et al.* 2007) in the genus *Crocus* (Iridacee) through the analysis of *rpoC1*, *matK* and *tmH-psbA* regions on 86 species of the genus *Crocus* and asserted the importance of barcoding as a promising technology.

Many marker-based studies have been performed for addressing the genetic diversity of *Crocus*. Moraga *et al.* (2009) analyzed the randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) marker profiles of 43 isolates of *C. sativus* to determine if this species is mono/polymorphic and assessed variability of saffron collected from different geographical areas. The results obtained from this study showed that all the clones collected from different geographical areas appeared as identical ones not only because of morphological characters but also at molecular level. In another study ISSR markers were used to characterize *C. sativus* and *Crocus cartwrightianus*, however, no differences were found thus confirming the earlier reports (Moraga *et al*. 2010). In contrast, molecular characterization based on RAPD markers revealed conside-

Table 1 Plant bioinformatics databases useful for saffron analysis.

Database	URL	Description
Saffron genes	www.saffrongenes.org	EST collection from saffron stigmas
NCBI Plant	www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html	Database on plant genomes
PlexDB	www.plexdb.org/	Data on plant expression
GRIN	www.ars-grin.gov/	Plant genetic resources
TAIR	www.arabidopsis.org	The *Arabidopsis* Information Resource
NASC	http://arabidopsis.info/	*Arabidopsis thaliana* Information
MATDB	http://mips.gsf.de/proj/thal/db/	*Arabidopsis thaliana* Information
NIAS db	www.dna.affrc.go.jp/database/	Database on rice
PlantCare	http://bioinformatics.psb.ugent.be/webtools/plantcare/html/	Database on *cis*-acting regulatory DNA elements in plants
EXPASY	www.expasy.org/links.html	Index to plant-specific databases
IRIS	www.iris.irri.org	International Rice Information System
EMBOSS	www.emboss.org	Sequence Analysis package
OPEN-BIO	www.open-bio.org	Sequence Analysis package
GMOD	www.gmod.org	Sequence Analysis package
TIGR	www.tigr.org/software	Sequence Analysis package
SBML	www.sbml.org	Metabolomics package
CROPFORGE	www.cropforge.org	Metabolomics package
ISCB	www.iscb.org	International Society for Computational Biology
PRINTS	www.bioinf.manchester.ac.uk/dbbrowser/PRINTS/index.php	Database of protein fingerprints
Phred/Phrap/Consed	www.phrap.org	Programme for EST assembly
Arachne	www.broad.mit.edu/wga/	Tool for assembling genome sequence
GAP4	http://staden.sourceforge.net/overview.html	Tool for sequence assembly
AMOS	www.tigr.org/software/AMOS/	Whole-genome shotgun assembly
KEGG	www.genome.jp/kegg/	Pathway maps and pathway modules
Metacyc	metacyc.org/	Database of non-redundant, experimentally elucidated metabolic pathways
Geneontology	www.geneontology.org	Database for annotation of gene sequences
CAP3	pbil.univ-lyon1.fr/cap3.php	Sequence assembly programme
GeneSpring	www.agilent.com/chem/genespring	Analysis of microarray data
CaARRAY	http://caarray.nci.nih.gov	Guides the annotation and exchange of array data
SWISS-2DPAGE	http://au.expasy.org/ch2d	Contains data on proteins identified on various 2-D PAGE and SDS-PAGE reference maps
Melanie	http://au.expasy.org/melanie	Two-dimensional electrophoresis (2-DE) gel analysis platform
Flicker	http://open2dprot.sourceforge.net/Flicker	An open-source stand-alone computer program for visually comparing 2D gel images
PDQuest	www.proteomeworks.bio-rad.com/html/pdquest.html	Software for analysis of 2D gels
PEDRo	http://pedro.man.ac.uk/	Configurable data entry tool for XML
Emowse	http://emboss.sourceforge.net/	Open Source software analysis package specially developed for the needs of the molecular biology
Mascot	www.matrixscience.com/	A powerful search engine that uses mass spectrometry data to identify proteins from primary sequence databases
SEQUEST	http://fields.scripps.edu/sequest	Correlates uninterpreted tandem mass spectra of peptides with amino acid sequences from protein and nucleotide databases
GOLM	csbdb.mpimp-golm.mpg.de/gmd.html	Database provides public access to custom mass spectra libraries, metabolite profiling experiments and other necessary information related to the field of metabolomics
MASSBANK	www.massbank.jp/index.html?lang=en	Database of comprehensive, high-resolution mass spectra of metabolites
Reactome	www.reactome.org/	A curated knowledgebase of biological pathways
Cytoscape	www.cytoscape.org/	An open source bioinformatics software platform for *visualizing* molecular interaction networks and *integrating* these interactions with gene expression profiles and other state data
Visant	visant.bu.edu/	Tool for biological networks and pathways

rable amount of genetic diversity among 10 elite saffron genotypes of Kashmir. The dendrogram based on molecular data divided the tested genotypes in two clusters at similarity coefficient of 44%, which showed a high level of genetic diversity between two clusters containing different genotypes (Imran *et al.* 2010). Alavi-Kia *et al*. (2008) used long terminal repeats (LTRs), a retrotransposon (RTN)-based marker study to analyze the genetic diversity and phylogenetic relationship in *Crocus* and to find the possible closest relatives of cultivated saffron from Iranian species of *Crocus*. Results of this study showed that except *C. sativus*, Iranian *Crocus* genus showed high diversity within and between species. In some cases, genetic variation was high among ecotypes of the same species from different geographical regions. These results also supported the possibility of Iranian *Crocus* species (*C. almehensis* and *C. mickelsonii*) as wild ancestors of saffron.

Apart from the whole genome sequencing projects, various efforts have been made for the generation of expressed sequence tags (ESTs) databases for different crops (Jantasuriyarat *et al.* 2005; Ramírez *et al.* 2005; Udall *et al.* 2006; Ashraf *et al.* 2009). ESTs provide an invaluable resource for analysis of gene expression associated with specific organs, growth conditions, developmental processes and responses to various environmental stresses and bridge the gap between genome sequence and gene function (Ashraf *et al.* 2009). In an effort to gain an understanding about the gene expression programs underlying accumulation of various apocarotenoids in *Crocus* stigmas, EST generation has been initiated recently (D'Agostino *et al.* 2007). The database is the first reference collection for the genomics of Iridaceae, for the molecular biology of stigma biogenesis and for the metabolic pathways underlying saffron secondary metabolism. D'Agostino *et al.* (2007) produced

6,603 high quality ESTs from a saffron stigma cDNA library and grouped these into 1,893 clusters, each corresponding to a different expressed gene. The complete set of raw EST sequences and their electopherograms are maintained in the *Saffron Genes* database [http://www.saffrongenes.org]. This allows users to investigate sequence qualities and EST structural features. The database structure consists of a main MySQL relational database and two satellite databases myGO and myKEGG. Further all the saffron genes were assigned a preliminary function using BLASTX to the UniprotKB/Swiss-Prot database. Gene ontology terms were assigned to the transcripts and it was found that in the molecular function ontology class, transcripts with catalytic and hydrolase activity were most represented while in case of the biological function class, the vast majority of the GO assignments corresponded to transport category. The saffron genes with enzymatic function were also mapped to KEGG databases so as to know which metabolic pathway they are involved in.

The abundance of ESTs in a particular contig is an indicative of mRNA abundance of that particular gene in the stigma tissue. Considering this, D'Agostino *et al*. (2007) looked for the contigs that composed of more than 20 ESTs. The highly expressed contigs were short chain hydrogenases, Cytochrome P450 sequences and several putative carotenoid metabolism enzymes like non-heme-β-carotene-hydroxylase, putative glucosyltransferase which is able to glycosylate crocetin *in vitro* (Moraga *et al.* 2004); putative isoprenoid GTases, one of which could represent the still missing enzyme responsible for the glycosylation of picrocrocin. Several contigs also encoded for putative transcription factors and the most abundantly expressed were Myb-like protein with high similarity to LhMyb (from *Lilium*, GenBank accession BAB40790), Myb8 (from *Gerbera*) (Elomaa *et al.* 2003) and Myb305 (from *Antirrhinium*) (Jackson *et al.* 1991. All these transcription factors are highly expressed in flowers.

This is a good beginning on a long way towards overall understanding of various metabolic pathways and their regulatory mechanisms that lead to the synthesis of biologically important metabolites in saffron. Thus bioinformatics helped in providing information regarding the probable function of the genes and the pathways they might have a role to play. Use of these genes for genetic engineering of such metabolic pathways would lead to enhanced levels of the important metabolites in genetically engineered saffron (Husaini *et al.* 2009).

In *Crocus*, the most valued metabolites are synthesised in stigma tissue and that too in developmental stage specific manner (Moraga *et al.* 2009). Thus characterization of the transcriptome of saffron stigmas is vital for throwing light on the molecular basis of flavor, color biogenesis, genomic organization and the biology of the gynoecium of spices in general and saffron in particular (Husaini *et al.* 2009). Some volatile and non volatile metabolites are also present in other *Crocus* tissues. This tissue and stage specific accumulation of various metabolites might be controlled by intricate regulatory networks of gene expression. Identifying these networks and the hierarchical relationship between them are vital to the understanding of biological systems. Moraga *et al.* (2009) used a combination of approaches to study the pattern of expression of several candidate genes and correlate that with volatile production and organoleptic characteristics of saffron during development. The pattern of accumulation of apocarotenoids in developing saffron stigmas was investigated by extracting stigmas corresponding to six different developing stages and analyzing the extracts by HPLC. The picrocrocin and crocin were detected in the early stages and increased rapidly during the following stages of development. In order to identify candidate genes encoding enzymes involved in volatile biosynthesis, *in silico* screening of the stigma cDNA database previously described (D'Agostino *et al.* 2007) was done and a comparison was drawn between the apocarotenoid content and the expression profiles. The results showed that during the development of *C. sativus* stigmas, the 1 deoxyxylulose 5 phosphate synthase (DXS), was expressed at all the stages while 3 hydroxy 3 methylglutaryl CoA reductase (HMGR) was expressed at low levels suggesting that DXS plays an important role in apocarotenoid accumulation. Also two putative terpene synthases were identified in the EST collection (TS1 and TS2) and each showed a different expression profile. The TS1 transcript was detected in all stages and slightly increased as the stigma developed. The transcript level of TS2 was undetectable during the early stages of stigma development and reaching a peak at preanthesis and anthesis thus suggesting the strong role of this enzyme in the biosynthesis of apocarotenoids. Similarly, transcript levels of two carotenoid biosynthesis genes *CsPSY* (phytoene synthase) and *CsPDS* (phytoene desaturase) increased in the red stage.

In another study (Castillo *et al.* 2005), accumulation of apocarotenoids was studied during stigma development followed by monitoring expression of some important genes of apocarotenoid biosynthetic pathway. It was observed that with the transition of yellow undeveloped to red developed stigmas, there was an accumulation of zeaxanthin accompanied by increased expression of phytoene synthase, phytoene desaturase and lycopene β cyclase. There was also a massive accumulation of β carotene hydroxylase and zeaxanthin cleavage dioxygenase transcripts. The expression of these two transcripts was also studied in relation to zeaxanthin and apocarotenoid accumulation in other Crocus species and only the relative levels of zeaxanthin in the stigma of each cultivar were correlated with the level of CsBCH transcripts. By contrast, the expression levels of CsZCD were not mirrored by changes in the apocarotenoid content, suggesting that the reaction catalyzed by the CsBCH enzyme could be the limiting step in the formation of saffron apocarotenoids in the stigma tissue.

Although a lot of literature can be found on botanical aspects of saffron, not much information on ecophysiological aspect of this species is available. Several environmental parameters affect flower induction in saffron amongst which temperature seems to play a pivotal role (Molina *et al.* 2005a, 2005b). Flower induction requires an incubation of the corms at high temperature (23-27°C), followed by a period of exposure at moderately low temperature (17°C) for flower emergence. There are evidences which show the critical importance of light and temperature in biological activities of plants including regulatory effects on dormancy period, vegetative and generative growth particularly flowering habit (Milyaeva and Azizbekova 1978; Halevy 1990). Transcriptome analysis of saffron plants subjected to different photoperiod and temperature regimes can throw light on the genes that get up or down-regulated and might lead to the identification of novel regulators which influence or lead to saffron flower initiation in a particular agro-climatic condition (Husaini *et al.* 2009). For example, comparison of environmental and management practices for saffron in Iran (Khorasan) and India (Kashmir) throw light on some basic climatic and topographic differences between the two regions viz., humidity, altitude, rainfall, soil-type and irrigation. The main similarities being in time of planting, harvesting and low temperatures during the growing season (Kafi and Showkat 2007). How these differences and similarities translate into gene expression can be known using DNA microarray technology and bioinformatics tools. The huge database generated by physiological, agronomic and gene expression studies can then be analyzed *in silico* to find agronomically important candidate genes in saffron (Husaini *et al.* 2009). This knowledge may also be used to specifically tailor saffron plants for new geographical areas with adaptability to specific climatic conditions. This will involve development of novel traits and agriculturally relevant characteristics through changes in gene regulation.

Recently a consortium, composed by 14 groups of 9 EU and non-EU countries has taken the responsibility of the creation and maintenance of the genetic variability of saffron and the European Commission has approved a project

on "Genetic Resources of Saffron and Allies (*Crocus* spp.): CROCUSBANK" to create, characterise and exploit a germplasm collection (bank) in *Crocus* species (Fernández 2007). This plant material can then be used in selection programmes and serve as sources of resistances to be transferred between saffron clones through appropriate breeding and biotechnological programmes. In realizing this objective, bioinformatics tools will be inevitable for locating these genes of resistance and agronomically important traits (Husaini *et al.* 2009). The removal of stamens and the hand separation of stigmas from saffron flowers are labour intensive and leads to the high cost of saffron stigmas (Tsaftaris *et al.* 2004). It is desirable to have saffron flowers, which do not form stamens, or even have carpels in place of stamens, thus doubling saffron production in a single flower while lowering the production cost. As C-class MADS-box gene function is essential for both stamen and carpel formation, Tsaftaris *et al.* (2005) characterized the expression of MADS-box genes in *Crocus* flowers using several molecular biology techniques, bioinformatics tools and database resources. Using a combination of conventional methods (5'- and 3'- RACE PCR) and new technologies (Rolling Circle Amplification RACE, and familyRCA-RACE) Tsaftaris *et al.* (2009) conducted a detailed study on the regulatory genes involved in flowering time and flower development of saffron. Such studies help in understanding and exploiting the molecular mechanisms that control flower development in crocus and in realization of the objective of producing flowers with carpels in place of stamens. Further, this knowledge can even be used in molecular medicine. Recently T and B-cell epitopes of Iranian *C. sativus* were mapped using bioinformatics tools and the predicted peptides were found useful for vaccine development (Hassan *et al.* 2008).

METABOLOMICS

Metabolomics is the analysis of the complete pool of small metabolites in a cell at any given time (Rhee *et al*. 2006). Metabolomics may prove to be particularly important in plants due to the proliferation of secondary metabolites. In a metabolite profiling experiment, metabolites are extracted from tissues, separated and analyzed in a high-throughput manner. Metabolic fingerprinting looks at a few metabolites to help differentiate samples according to their phenotype or biological relevance (Edwards and Batley 2004). Bioinformatics tools of metabolomics help in the identification and characterization of a broad range of metabolites through reference to quantitative biochemical analysis (Edwards and Batley 2004). In this aspect GOLM metabolome database and the MASSBANK database have been extensively used. Various databases like KEGG, Reactome, MetaCyc and GO-ontology further help to know about the biochemical pathways in which the identified metabolites act and perform role. Biogenesis of the three major metabolites, crocetin glycosides, picrocrocin, and safranal, which are responsible for saffron colour, bitter taste and aroma, takes place from the oxidative cleavage of the carotenoid zeaxanthin (Bouvier *et al.* 2003). This step results in the formation of crocetin dialdehyde, and the final product crocetin glycoside (Moraga *et al.* 2004); and two identical β-ionone molecules, hydroxyl β-cyclocytral, which is thought to be converted by an UDP-glucosyltransferase to picrocrocin and safranal. Metabolomic studies of saffron enzymes involved in flavonoid glucosylation (flavonoid glucosyltransferases; Rubio *et al.* 2009) and carotenoid biosynthesis (lycopene β-cyclase, Ahrazem *et al.* 2009) have the potential to reveal the dynamics of these pathways. Analysis of apocarotenoid content of *Crocus* at various stages of stigma development revealed that young yellow stigmas contained very low levels of crocetin, crocins, and picrocrocin and mainly contained unidentified compounds with maximum wavelengths around 250 nm that reached the highest levels in the orange stage. The apocarotenoid picrocrocin and crocins were detected early in the orange stage and increased rapidly during the following stages of stigma development and reaching at their maximum in the red stage. Also the glycosylated products of crocetin reached the highest levels in the red stage. The accumulation of crocins of higher glucose content agreed with the expression patterns observed for UGTCs2, a glucosyltransferase enzyme involved in crocin and crocetin glucosylation in C. sativus stigmas (Rubio *et al*. 2004). The levels of picrocrocin began increasing during the orange stage and reached the highest levels at anthesis, the stage of flower development when stigmas are collected for saffron preparation and which is characterized by high levels of the volatile safranal (Carmona *et al.* 2007).

In addition to the apocarotenoids, saffron also contains volatile compounds and it has been estimated that around 150 such compounds are present in saffron out of which only one third have been identified (Winterhalter and Straubinger 2000). Further, different and characteristic profiles of volatile compounds have been obtained for each developmental stage of saffron. For example, In the yellow stage, low levels of volatiles were produced and the fatty acid derivatives predominated while in the orange stage, carotenoid derivatives were detected in addition to the fatty acid derivatives. In the red stage, the volatiles derived from carotenoids accumulated to high levels, and β-cyclocitral, generated by the cleavage of β-carotene reached its maximum levels suggesting that β-carotene contributes in this stage to the pool of crocin and crocetin. At the scarlet stage, right before anthesis, the volatile propanoic acid, 2-methyl-2,2-dimethyl-1-(2-hydroxy-1-methylethyl) propyl ester accumulated at high levels, but their levels decreased at anthesis, when monoterpenes and carotenoids reached their maximum levels. Interestingly, safranal was not among the main volatiles generated in the fresh tissue where high levels of picrocrocin were observed. In contrast, safranal has been considered as the major aroma component in saffron, comprising as much as 60-70% of the essential oil content (Alõnso *et al*. 1996; Tarantilis and Polissiou 1997), suggesting that this compound is most probably generated by picrocrocin degradation during the dehydration process of the stigma (Raina *et al*. 1996). Among the monoterpenes, linalool was emitted at high levels at anthesis, and is commonly described as responsible for fresh and floral odours (Dobson 1993; Knudsen *et al*. 1993). In the postanthesis stage, the fatty acid derived volatiles became the main volatile compounds together with the degradation product 4-oxoisophorone, suggesting that products of senescence may differ from those actively produced in mature stigmas, which would help to make the flowers less attractive for pollinators. This reduced attractiveness is hypothesized to guide pollinators to the unpollinated and mature flowers, and thus increase the reproductive success of the plant (Negre *et al*. 2003; Theis and Raguso 2005).

Keeping this in view studies should be focussed towards the identification of various other saffron metabolites too which may also lead to the identification of new compounds having important medicinal properties and can be used as novel or improved phytotherapeutic agents.

COMPARATIVE GENOMICS

Comparative studies would help in understanding the complexity in various metabolic pathways including carotenoid biosynthesis in saffron. Carotenoid biosynthesis and its regulation have been studied in various plant species like *Zea mays* (Harjes *et al.* 2008), *Daucus carota* (Clotault *et al.* 2008), tomato (Giuliano *et al.* 1993), etc. These plants are studied in much more detail and more genomic information is available for them as compared to *Crocus*. Therefore, we can compare the genomic information of Crocus with these crops and determine the differences in carotenoid synthesis. Many enzymes involved in carotenoid biosynthetic pathways belong to gene families (Gallagher 2004). How members of a gene family differ in their function would further help in understanding these complex pathways. This would throw light on how carotenoid biosynthesis is regulated in

stage and tissue specific manner in *Crocus*, and give an overview of evolution of these genes and pathways.

The first International Symposium on Saffron Biology and Biotechnology, held in Albacete, Spain (2003) stressed the need for the creation of a bank of germplasm and gene banks in saffron. In 2005, a programme was launched by the European Commission for the conservation, characterisation, collection and utilisation of genetic resources in agriculture, AGRI GEN RES and a joint consortium with partners from 9 countries (CROCUSBANK) was constituted with two main goals (Fernández 2007; de los Mozos-Pascual *et al.* 2010a). The first being the collection and reproduction of saffron bulbs from all countries that cultivate saffron; and second, the collection of saffron allies for research into the taxonomy, evolution, genetics, physiology, ecology and agronomy of the genus. Currently the project is under progress and 384 accessions of saffron and wild crocuses are being preserved, multiplied and partially characterised in the Bank of Plant Germplasm of Cuenca (Spain) (de los Mozos-Pascual *et al.* 2010a). Preliminary characterisation of 50 saffron accessions from Azerbaijan, France, India, Iran, Italy, Morocco, New Zealand, Spain and Turkey has been done for characters related to phenology, floral morphology and saffron production. In addition preliminary DNA characterisation on 25 accessions has also been performed using PCR-based molecular markers (de los Mozos-Pascual *et al.* 2010b). Here bioinformatics tools can help in appreciation of the extent of the diversity of various geographic and/or genetic groups of cultivated saffron. Molecular-based trees can be constructed using software like CLUSTAL-W, MultAlign to infer relationships among groups and accessions (Husaini *et al.* 2009).

INTEGRATED APPROACH

The above mentioned approaches can help in exploring the biology of metabolite production in *Crocus* and their tissue specific accumulation. However, if used together, these approaches and technologies hold the potential of revolutionizing the biology of saffron. In saffron the overall metabolic process which leads to the synthesis of carotenoid compounds is a coordinated effort of many individual pathways. Further, the metabolite composition of saffron is different at different stages. Also other flower parts like sepals and petals contain a different set of metabolites. In this context, parallel analysis of transcript and metabolite profiling can reveal unexpected gene to metabolite networks by correlating the expression pattern of all genes with accumulating pattern of all metabolites (Urbanczyk-Wochniak *et al.* 2003). This would lead to the identification of new genes, unravelling of metabolic pathways and developing of correlation networks which will help to understand how different genes and their expression pattern affect the production and accumulation of various apocarotenoid compounds. This knowledge will be of immense use to remodel the carotenoid biosynthetic pathways so as to arrive at enhanced accumulation of various biologically active and important metabolites. There are many bioinformatics softwares available for integration of such data and generation of correlation networks, for example, Cytoscape, Visant, etc.

Integration and structured interrogation of metabolome and transcriptome datasets would provide the basis for the integration of genome and phenome data. Linking gene expression, protein sequence and protein structure data will integrate genomics, transcriptomics and proteomics. Further incorporation of metabolomic data would create the foundation for advanced knowledge-bases and help address a range of biological questions.

CONCLUSION

Saffron is genetically a rich crop as far as its use as spice and medicine is concerned. Not much effort has been made so far towards exploring its potential and its subsequent improvement. With growing need of this crop, the call for the day is integration of multiple biotechnological approaches and use of bioinformatics tools for analysing and integrating the data so as to acquire maximum output towards genome prospecting of this crop. While transcriptomics, proteomics and metabolomics would generate data, bioinformatics would provide glue for integration of these diverse areas so as to provide more comparative, connected and holistic view about the biology of saffron. This will further pave way to design the strategy for improvement of this crop in various aspects.

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How to reference: Author name(s) (2010) Title of chapter. In: Husaini AM (Ed) Saffron. Global Science Books, UK, pp. X-XX

Expression Analysis of Flower MADS-box Genes in Saffron *Crocus* (*Crocus sativus* L.) Supports a Modified ABCDE Model

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ABSTRACT

Crocus sativus L. is a monocot triploid species, member of the family Iridaceae, and is considered to be the highest priced spice in the world. It is cultivated for its flowers and more specifically for its red stigmas. The flower of *Crocus* is bisexual and it is sterile. The dry form of stigmas constitutes saffron. In order to uncover and understand the molecular mechanisms controlling flower development in cultivated *Crocus* and its relative wild progenitor species, and characterize a number of *Crocus* flower mutants we have cloned and characterized different full length cDNA sequences encoding MADS-box transcription factors belonging to the different ABC and E-class MADS box proteins. Herein, we review the isolation of *Crocus* MADS box genes and primarily discuss their expression patterns in leaves and the four flower organs: outer tepals, inner tepals, stamens and carpels. Expression analysis of the isolated MADS box genes support the hypothesis that a modified ABCDE model in the flower of *Crocus* is responsible for the development of the different *Crocus* flower organs and the transformation of the sepals and petals into tepaloid organs, designated outer tepals and inner tepals, respectively.

Keywords: flower, gene expression, MADS-box genes, monocots

CONTENTS

INTRODUCTION

Flowering plants or angiosperms represent one of the most successful and diverse groups of organisms on the planet, with more than 250,000 extant species in the wild and thousands more varieties generated by horticulturists through hybridization and other breeding methods. Although angiosperms such as orchids, roses and snapdragons have very characteristic flowers, most flowers contain four organ types and they have highly conserved developmental molecular mechanisms (Krizek and Fletcher 2005). The majority of flowers have four types of floral organs: two outer whorls of sterile organs, the sepals and petals (also known as the perianth), and two inner whorls of fertile organs, the male stamens and female carpels, with the carpels positioned centrally. Although the main characteristics of angiosperm flowers are generally conserved, the vast morphological diversity suggests a high degree of plasticity in the genetic control of floral development. Variation is observed in every aspect of floral architecture, including phyllotaxy, merosity, floral symmetry and floral organ identity. In-depth analyses of model species such as *Arabidopsis thaliana* and *Antirrhinum majus* have contributed significantly to our understanding of the genetic pathways that control these morphological components. By using this work as a foundation for comparative studies, a picture is gradually coming into focus of how alterations in floral genetic programs have contributed to the evolution of floral architecture.

Forward mutagenesis studies of that two model species uncovered an intriguing series of homeotic floral mutants (Komaki *et al*. 1988; Bowman *et al*. 1989; Coen and Meyerowitz 1991). In both taxa, the mutants appeared to fall into similar classes named A, B and C: mutations that affected sepal and petal identity were placed into what was termed the A-class; those that affected petal and stamen identity, the B-class; and those that affected stamen and carpel identity, the C-class. For instance, B mutants exhibited the transformation of petals into sepals and stamens in carpels (Bowman *et al*. 1989; Carpenter and Coen 1990). Analysis of double and triple mutants (Bowman *et al*. 1991) suggested a simple and elegant model that explained the major aspects of genetic interactions among the loci; this became known as the ABC model (Coen and Meyerowitz 1991). Fundamentally, the ABC model holds that the overlapping domains of three classes of gene activity, referred to as A, B and C, produce a combinatorial code that determines floral organ identity in successive whorls of the developing flower. Another critical component of the ABC program is that A and C functions are mutually exclusive (Bowman *et al*. 1991), such that elimination of C gene activity causes

Table 1 Homologues of the major A, B, C, D, E classes of MADs-box genes from *Crocus*, the two dicots model species *Arabidopsis* and *Antirrhinum* and the monocot model *Oryza*. The number of crocus homologues for each class of MADs-box gene obtained from *Crocus* and method followed to obtain each sequence is also indicated.

Gene class	*Arabidopsis*	*Antirrhinum*	*Oryza*	*Crocus*	Number of isolated *Crocus* homologues	Cloning method (*Crocus*)	Reference (*Crocus*)
A	*APETALA1*	*SQUAMOSA*	*nk*	*nk*	-	-	-
B	*APETALA3*	*DEFICIENS*	*SUPERWOMAN1*	*CsatAP3/DEF*	2	5′ 3′ RACE	Tsaftaris *et al.* 2006
	PISTILLATA	*GLOBOSA*	*OsMADS2, OsMADS4*	*CsatPI/GLO*	5	5′ 3′ RACE	Kalivas *et al.* 2007
C	*AGAMOUS*	*FARINELI*	*OsMADS3*	*CsatAG1*	2	5′ 3′ RACE	Tsaftaris *et al.* 2005
D	*SEEDSTICK*	*nk*	*OsMADS13*	*nk*	-	-	-
E	*SEPALLATA1, 2, 3*	*DEFH49, 72*	*LHS1, OsMADS5, 34*	*CsatSEP3*	4	famRCA-RACE	Tsaftaris *et al.*, unpublished
	AGAMOUS-LIKE6	-	-	*CsatAGL6*	2	5′ 3′ RACE	Tsaftaris *et al.*, unpublished
			RAP1	*Csat AP1/FUL*	3	5′ 3′ RACE and RCA-RACE	Tsaftaris *et al.* 2004

nk: not known

the A domain to expand and *vice versa* (Drews *et al*. 1991; Gustafson-Brown *et al*. 1994).

Later, based on protein - protein interactions, the ABC model was extended to the ABCDE model (Theissen and Saedler 2001). Whereas the E-class genes together with the B and C genes control stamen formation, the C- and E-class genes regulates carpel formation and the D-class genes are involved in ovule development. According to the model, two dimers of each tetramer recognize two different DNA sites (termed CArG-boxes) on the same strand of DNA, which are brought into close proximity by DNA bending.

With the exception of APETALA2, all of the organ identity genes identified to date are members of the pan-eukaryotic MADS transcription factor family (Becker *et al*. 2003; Messenguy and Dubois 2003). MADS-box genes are characterized by the highly conserved MADS-box domain and can be divided into the type I and type II main lineages that are present in plants, animals and fungi (Theissen *et al*. 2000; De Bodt *et al*. 2003b). These lineages differ in the amino acid sequence of the MADS-box as well as in the domain structure of the predicted protein. Most of type II proteins exhibit a typical MIKC-structure where the MADS-domain is followed by a short I (intervening) domain, a well conserved K (keratin-like) domain and a variable C-terminal region, while type I proteins lack the K-domain, forming a structure of a MADS-box followed by a rather undefined and length-variable C-domain (De Bodt *et al*. 2003a).

Although monocot flowers contain stamens and carpels, they differ from eudicot flowers in the type of organs that are present in the outer whorls. Liliaceae family members often have two outer whorls of showy petal-like tepal organs, whereas grass flowers have paleas, lemmas and lodicules in place of sepals and petals. A modified ABC model in which B function is present in whorls 1, 2 and 3 has been proposed to explain the presence of tepals in Liliaceae flowers (van Tunen *et al*. 1993b). This is supported by the observation of B-class *AP3/DEF*-like and *PI/GLO*-like gene expression in the outer three whorls of tulip flowers (Kanno *et al*. 2003), and the absence or low expression of *AP3/DEF*-like genes in the outermost whorl of other monocots that produce distinct sepals and petals (Ochiai *et al*. 2004). Studies in *Zea mays* and *Oryza sativa* indicate that B-class genes have similar roles in grass and eudicot flowers (Whipple *et al*. 2004). Loss of the single *AP3/DEF*-like gene in maize *SILKY 1* and *O. sativa SUPERWOMAN 1*, results in replacement of lodicules by paleas or lemmas, or palea-like organs, respectively, and the replacement of stamens by carpels (Ambrose *et al*. 2000; Nagasawa *et al*. 2003). These homeotic transformations are similar to those observed in *Arabidopsis* and *Antirrhinum* B-class mutants, indicating that paleas and lemmas are homologous to sepals, and lodicules are homologous to petals. Maize contains two potential C-class *AG/PLE*-like genes (*ZAG1* and *ZMM2*), but redundancy makes it difficult to determine their exact roles (Mena *et al*. 1996). Mutations in ZAG1 affect floral determinacy but not organ identity. Carpels are still produced in rice plants with reduced expression of the *AG/PLE*-like gene *MADS3* (Kang *et al*. 1998), indicating that other factors are required for carpel specification in grasses. The *YABBY* gene *DROOPING LEAF* (DL) is one such factor (panel b), as mutations in DL result in complete homeotic transformations of carpels into stamens (Nagasawa *et al*. 2003; Yamaguchi *et al*. 2004). Although A-class *AP1/SQUA*-like genes have been identified in maize and rice, their roles in flower development are not well defined.

THE FLOWER OF *CROCUS*

Studying flower development and flower organs formation is not only significant for improving our understanding of basic regulatory mechanisms of flower initiation and organ identity, but could have practical applications in crops cultivated for their flowers. *Crocus*, an autumn flowering geophyte of the Mediterranean region, is an example of such a crop with flowers of economic importance. *Crocus* is a monocot triploid species belonging to the Iridaceae family, whose red stigmatic styles constitute saffron, a popular food additive with delicate aroma and attractive colour. Saffron has also medicinal properties and is used in the colouring industry. The flower of *Crocus* is bisexual and as sterile it has an exclusively vegetative propagation forming only 3-4 cormlets each season. Perianth consists of six petaloid tepals; three tepals in whorl 1 (outer tepals) and three tepals in whorl 2 (inner tepals, **Fig. 1B**). Androecium consists of three distinct stamens and the gynoecium consists of a single compound pistil with: three carpels, a single three-branched style, and an inferior ovary. Several phenotypic flower mutants have been described, such as flowers with larger numbers of styles and stamens as well as flowers without stamens (Grilli Caiola *et al*. 2004). *Crocus* blooms only once a year and is hand harvested. After mechanical separation of tepals, the stigmas are hand separated from carpels and dried. The size and the amount of individual stigmas collected from each flower influence total yield and quality of saffron. Between 70,000 and 200,000 flowers are needed to produce 1 kg of dried saffron, which equates 370–470 h of work. Consequently, the cultivation of this crop for its flowers and specifically its stigmas is very labour-intensive leading to high costs (Tsaftaris *et al*. 2004). Thus, understanding flower development in *Crocus* could reveal ways to increase yield and lower production costs since flower and more specifically isolated stigmas comprise the valuable commercial part of the plant.

Towards this goal we have cloned and characterized representatives of all MADS-box genes from *Crocus*, and very recently we obtained the *A*PETAL*A2* homologue from *Crocus* (unpublished data).

In order to isolate genes involved in flowering and flower development we developed and improved new methods and protocols such as the Rolling Cycle Amplification RACE (RCA-RACE) (Polidoros *et al*. 2006; Tsaf-

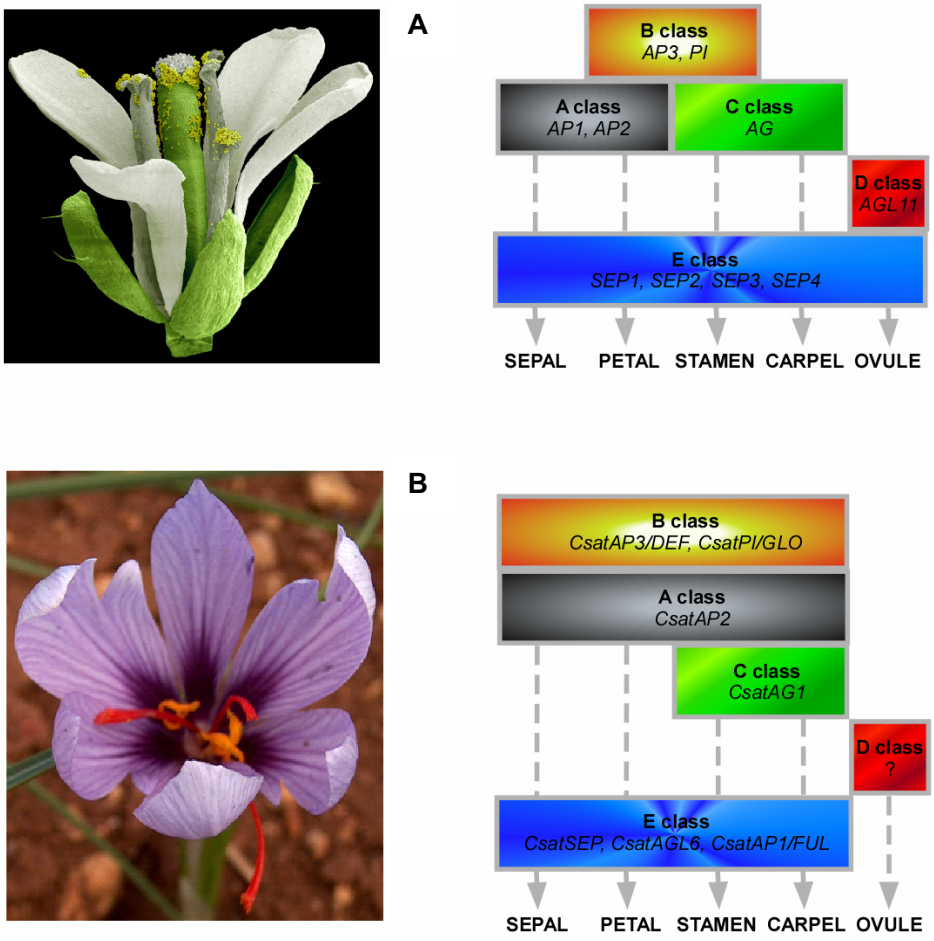

Fig. 1 (**A**) The classic ABC model (Coen and Meyerowitz 1991) for floral organ identity in *Arabidopsis* is shown as orange, grey and green boxes. Based on recent additions to the ABC model, D- and E-class genes are shown as red and blue boxes. (**B**) The modified ABC model for floral organ identity in *Crocus*.

taris *et al.* 2007, 2010) and Family RCA-RACE (Kalivas *et al.* 2010). Applying the above methods allowed us to isolate full length cDNAs and genomic sequences of target genes playing critical roles in flower formation. In a previous review of MADS-box genes involved in the flower of *Crocus* we described their comparative structural and phylogenetic relationships (Tsaftaris *et al.* 2007). **Table 1** displays homologues of the major B, C, and E-classes of MADS-box genes from *Crocus*, the two dicots model species *Arabidopsis* and *Antirrhinum* and the monocot grass model crop rice. The number of *Crocus* homologues for each class of MADS-box sequences obtained from *Crocus* and the method followed to obtain each *Crocus* sequence is also indicated.

EXPRESSION OF *CROCUS* MADS-BOX GENES IN FLOWER

The expression pattern of all the isolated MADS-box genes from *Crocus* in leaves, flowers and in four different flower organs: outer tepals, inner tepals, stamens and carpels was compared by RT-PCR. More specifically the expression pattern of the isolated B, C and E-class MADS-box genes from *Crocus* was examined using cDNA synthesized from leaves, whole flowers and the flower organs sepals, petals, stamens, and carpels.

B-class MADS-box genes

B-class genes have been isolated from several monocot species and their function has been examined in mutants, such as the si1 of maize (Ambrose *et al.* 2000) that exhibit homeotic conversions of stamens into carpel-like and lodicules into palea/lemma-like organs. In rice, the expression patterns and mutant analysis of an *AP3*-like *SPW1* and two *PI*-like *OSMADS2* and *OSMADS4* genes provide supportive evidence for conservation of B-function as predicted from the ABC model in this plant (Kang *et al.* 1998; Moon *et al.* 1999; Nagasawa *et al.* 2003). The above data point to a conserved role for B-class proteins between dicots and monocots of the grasses family. There is also enough evidence to suggest that B-function is conserved in other monocots. The Asparagus AODEF is expressed exclusively in whorls 2 and 3 during the hermaphrodite stages of flower development and its expression is detected in the respective organs of the male but is reduced in the female flowers (Park *et al.* 2003). The lily LMADS1 protein is detected only in petals and stamens although the gene is expressed in all 4 whorls. Additionally, a truncated *LMADS1* lacking the MADS domain, when expressed ectopically in Arabidopsis, can confer a negative dominant phenotype resembling ap3 mutants that have petals transformed into sepal-like, and stamens into carpel-like structures (Tzeng and Yang 2001).

It has been suggested that probably a conserved role of B-class genes in monocots and dicots is the specification of male reproductive organs, while their role in the formation of lodicules in grasses or tepals in Liliales and Asparagales may be not similar to that in formation of petals in eudicots, since homology of these organs remains controversial (Kramer and Irish 1999; Ambrose *et al.* 2000). However, it has also been suggested that formation of petaloid organs in whorl 1 in several eudicots could be due to the transference of the B-function in this whorl (Baum and Whitlock 1999). The same has been proposed as explanation for the formation of tepals in lilies and tulips (Theissen *et al.* 2000). Expression of B-class genes in whorl 1 is not an uncommon phenomenon in monocots, since it can be observed (especially when in addition to northern analysis, sensitive PCR

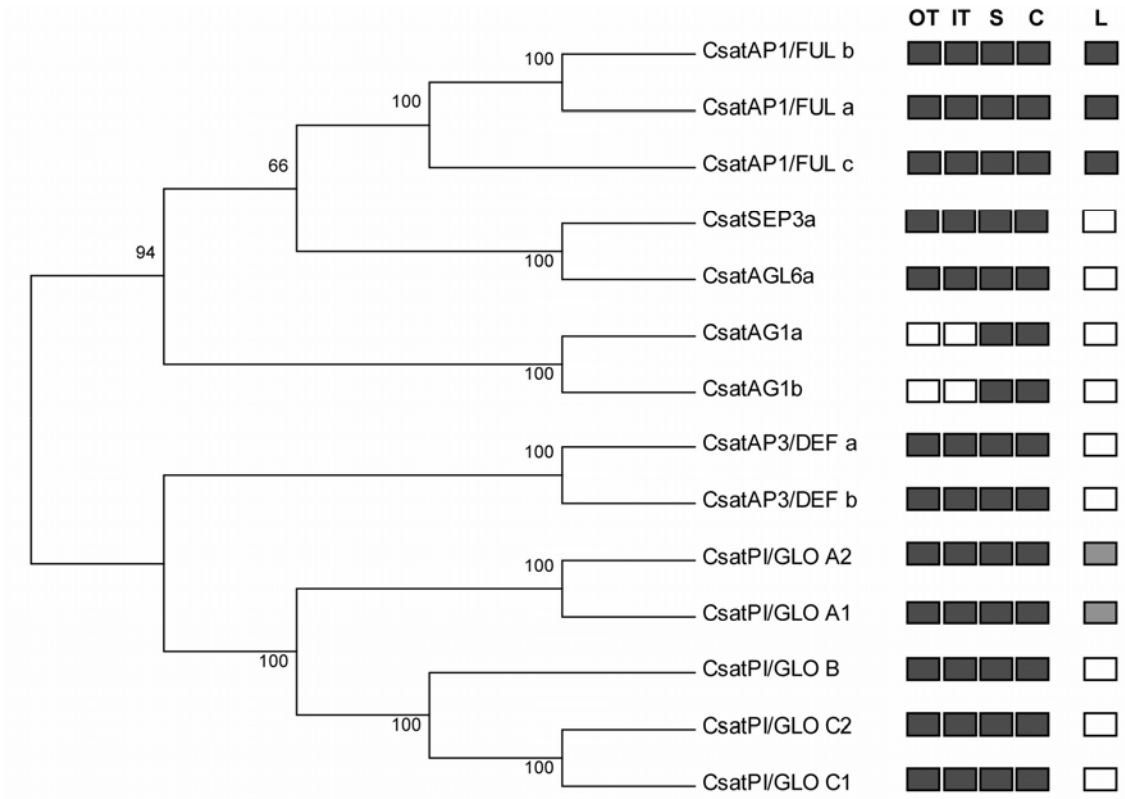

Fig. 2 Phylogeny and expression patterns of the major classes of MADS-box proteins from *Crocus*. The tree was generated by the Neighbor-Joining method using the p-distance correction. Numbers next to the nodes are bootstrap values from 1000 replications. The grey box indicates the identification of the MADS-box transcripts in the flower organs: outer tepals (OT), inner tepals (IT), stamens (S), carpels (C) and in the leaves (L).

techniques are used) in several species (Johansen *et al.* 2002). However, there are examples where expression in whorl 1 was not followed by accumulation of active protein and did not support presence of B-function, as in lily (Tzeng and Yang 2001). In tulip the AP3-like genes *TGDEFA* and *TGDEFB*, as well as, the PI-like *TGGLO*, are expressed in whorls 1, 2 and 3 (Kanno *et al.* 2003). Presence of both, *AP3*-like and *PI*-like proteins in whorl 1 should be a strong indication to explain formation of petaloid organs since ectopic expression of both *AP3* and *PI* in whorl 1 in *Arabidopsis* resulted in the conversion of sepals into petals demonstrating that these genes are sufficient to provide B-function in flowers (Krizek and Meyerowitz 1996). Thus, Kanno *et al*. (2003) provided evidence to support the modified ABC model that was proposed by van Tunen *et al*. (1993b) to explain the flower morphology in tulip. Similar results were obtained in this study for the formation of *Crocus* flower suggesting that the power of the modified ABC model extends in Asparagales. Our data show that the isolated AP3-like *CsatAP3/DEF* sequences and *CsatPI/GLO* are expressed in whorl 1 and may be involved in the homeotic transformation of sepals into tepals. More specifically the experiments revealed the presence of *CsatPI/GLOA* transcripts both in flowers and weakly in leaves while the *CsatPI/GLOB* and *CsatPI/GLOC* transcripts were present only in flowers (**Fig. 2**).

The expression analysis of *CsatAP3/DEF*, the other B-class MADS box gene in *Crocus*, in leaves and flowers revealed the presence of both transcripts only in flowers and not in leaves (**Fig. 2**). The RT-PCR experiment performed with cDNA synthesized from outer tepals, inner tepals, stamens and carpels resulted in the identification of both transcripts in all mature flower parts (Tsaftaris *et al.* 2006). Thus as anticipated from the ABC model and found in other plant species with tepal formation, both B-class genes *CsatAP3* and *CsatPI* extend their expression to whorl1 leading to tepal formation in this whorl instead of sepals.

It is conceivable that even though our results provide supportive evidence for the relevance of a modified ABC model in outer tepal formation in *Crocus*, much has to be done in order to understand flower formation in *Crocus* and other Asparagales species. Further experiments are underway to understand homeotic transformations in *Crocus* flowers and to characterize and possibly exploit the numerous flower mutants (lack of stamens, multiple flower organs, etc.) frequently observed in fields cultivated with this asexually propagated crop.

C-class MADS-box genes

C-class genes, such as Arabidopsis *AGAMOUS* (AG) and *Antirrhinum PLENA* (PLE), play important roles in the specification of stamen and carpel identity, the control of floral meristem determinacy, and the negative regulation of A-class gene activity (Coen and Meyerowitz 1991). The rice genome contains two C-class genes, *OsMADS3* and *OsMADS58*, that arose through gene duplication (Kang *et al.* 1998; Yamaguchi *et al.* 2006). Molecular genetic studies on these two rice C-class genes have revealed further functional diversification of duplicated MADS-box genes (Yamaguchi *et al.* 2006). *OsMADS3* and *OsMADS58* are expressed in the stamen and carpel whorl, like typical eudicot C-class genes, but their temporal patterns of expression differ from each other. *OsMADS3* is expressed only in the presumptive region of stamen and carpel primordia just before the initiation of these organs, whereas *OsMADS58* is expressed in the stamen and carpel whorls before initiation and during the development of stamen and carpel primordia. In an insertional knockout mutant of *OsMADS3*, stamens are homeotically transformed into lodicules, whereas carpels develop almost normally. Thus, *OsMADS3* plays a predominant role in stamen specification. By contrast, RNA-silenced lines of *OsMADS58* develop indeterminate flowers that reiterate a set of floral organs including lodicules, stamens, and abnormal carpel-like organs. In these reiterated flowers, stamens develop almost normally, but carpels are morphologically abnormal. Thus, *OsMADS58* has a critical function both in the establishment of floral meristem determinacy and in normal carpel development. These results indicate that the original functions of C-class genes have

been partitioned into two paralogous genes, *OsMADS3* and *OsMADS58*, during the evolution of rice. The mechanism underlying this functional diversification of C-class genes in rice may be partially explained by variation in the temporal expression of the two C-class genes. For example, *OsMADS3* is down-regulated in the floral meristem before meristem activity is terminated, whereas expression of *OsMADS58* is maintained even after carpel initiation. The predominant function of OsMADS58 in floral meristem determinacy cannot be explained by its expression profile alone, however, because *OsMADS58* is expressed uniformly in both whorl 3 and whorl 4. Another possibility is that differences in the proteins that interact with the two MADS-domain proteins may have led to this functional diversification. That is, OsMADS3 and OsMADS58 may interact specifically and independently with different factors that are preferentially expressed in whorl 3 and whorl 4, respectively. The exact molecular mechanism underlying the functional diversification of OsMADS3 and OsMADS58 during the evolution of rice remains an interesting subject for future study. Another role of the C-class genes in rice, revealed by functional studies, is the control of lodicule positioning (Yamaguchi *et al.* 2006). In both a loss-of-function line of *OsMADS3* and an RNA-silencing line of *OsMADS58*, ectopic lodicules form at the palea side of whorl 2, leading to a radially symmetric arrangement of lodicules, whereas lodicules develop only at the lemma side of whorl 2 in wild type. The spatial expression patterns of *OsMADS3* and *OsMADS58* are also asymmetric in the wild-type flower; that is, the two genes are down-regulated at the lemma side of whorl2 where lodicules develop, but are up-regulated in the region adjacent to the palea. These observations indicate that C-class genes in rice negatively regulate lodicule development at the palea side in the wild-type flower.

The expression analysis for the isolated C-class *CsatAG1* gene in *Crocus* organs revealed the presence of the transcript only in flowers and more specifically restricted in the reproductive parts of the flower: stamens and carpels and not in the perianth. Thus the C-class *CsatAG1*, respects the ABC model where C-class *AGAMOUS* is expressed only in the reproductive parts of the flower (**Fig. 2**; Tsaftaris *et al.* 2005). Furthermore this expression pattern, in agreement with the ABC model of floral organ development, provides the basis for classification of *CsatAG1* in the C-class MADS-box genes.

E-class MADS-box genes

Three groups of genes are included in the E-class MADS-box gene isolated from *Crocus*, namely *CsatA*PETAL*A1/FRUITFUL*, *CsatSEPALATA* and *CsatAGAMOUS LIKE-6*. Originally, many laboratories working with monocots including ours, described the homologues *AP1/FRU* sequences identified and described as *A*PETAL*A1* or *FRUITFUL*. Despite the homologies between *A*PETAL*A1* and *FRUITFUL* all the isolated sequences from monocots so far belong to the FRUITFUL sub-group and no APETALA1 function was described in all monocots so far. Thus we describe as E-class gene the *CsatAP1/FRU*, were its FRU-like sequence belongs.

The results from *Crocus* showed that transcripts of the three isolated genes *CsatAP1/FULa*, *CsatAP1/FULb*, and *CsatAP1/FULc* are present in leaves, as well as, in flowers of *Crocus* (**Fig. 2**). Expression analysis performed in sepals, petals, stamens, and carpels resulted in the identification of the *CsatAP1/FULa*, *CsatAP1/FULb*, and *CsatAP1/FULc* transcripts in all tissues examined (Tsaftaris *et al.* 2004). In *Arabidopsis*, expression of *AP1/FUL* occurs specifically in the tissues and at the developmental stage in which floral fate is assumed. In the flower, expression of *AP1/FUL* is restricted to petals and sepals. In contrast, the three isolated *CsatAP1/FUL* genes from *Crocus*, are *AP1/FUL*-like MADS-box genes expressed in vegetative as well as in all floral tissues of the plant. There are also several examples of MADS-box genes belonging to different homeotic types that are expressed in vegetative tissues and have different functional roles (Zhang and Forde 1998; Alvarez-Buylla *et al.* 2000; Gocal *et al.* 2001; Skipper 2002; van der Linden *et al.* 2002). The similarities in expression pattern of many monocot *AP1/FUL*-like MADS-box genes, including the rice *OsMADS18*, the barley *BM3*) (Schmitz *et al.* 2000) and the three isolated in this study *Crocus CsatAP1/FUL* genes, as well as other such genes with floral and vegetative expression may indicate a novel class of MADS-box genes in monocots, and possibly reflect a novel, yet unidentified role of the corresponding proteins as transcriptional regulators in these species.

The expression analysis of *CsatSEP3a*, a second E-class MADS box gene in *Crocus*, in leaves and flowers revealed the presence of the transcript only in flowers and not in leaves. Furthermore the *CsatSEP3a* transcripts were also detected in sepals, petals, stamens and carpels (**Fig. 2**, Tsaftaris *et al.* submitted).

Unlike the functional-structural conservation, the expression pattern of *SEP*-like transcripts differs between monocots and eudicots. Within monocots, *SEP*-like genes have been most intensively studied in grasses, including the important cereals maize and rice (Tzeng *et al.* 2003). Similarly to other monocots, expression of the isolated *SEP3*-like genes of *Crocus* was detected in all four whorls of flower organs. This pattern of extended expression of E-class genes together with B-class genes reported previously (Tsaftaris *et al.* 2006; Kalivas *et al.* 2007) is compatible with tepal formation in whorl 1. *Muscari armeniacum* is another member of Asparagales that has petaloid organs in the outer two whorls and expression of B-class genes extended to whorl 1, similarly to *Crocus* (Nakada *et al.* 2006). Thus, the extended expression of B- and E-class genes in whorl 1 fits the modified ABCE model proposed to explain tepal formation in tulip and other nongrass monocots (van Tunen *et al.* 1993a; Kanno *et al.* 2003; Kanno *et al.* 2007).

Expression analysis of a third subgroup of E-class MADS-box gene isolated in our laboratory, *CsatAGL6a*, indicated expression in flowers but not in leaves and all four flower organs examined (**Fig. 2**). The Arabidopsis *AGL6* genes named *AGL6* and *AGL13* despite their close relation as a result of their very recent duplication show quite different expression patterns. While *AGL6* is expressed in all four types of floral organs (Mouradov *et al.* 1999), the expression of *AGL13* is restricted to ovules (Rounsley *et al.* 1995). But none of them is expressed in leaves, too. In other monocots such as *Asparagus officinalis* the *AGL6*-like *AOM3* is expressed not only in flower organs, but also in the different meristems present on the apical region of the shoot during the flowering season (Losa *et al.* 2004). *ZAG3* and *ZAG5* are maize AGL6-like genes and their expression was found to be floral-specific and present in both male and female maize inflorescences (Mena *et al.* 1995). *ZAG3* is expressed in carpels, but not in stamens, and in the sterile floral organs but not in glumes (Mena *et al.* 1995). In that respect it is interesting the absence of expression of *AGL6* transcripts in a *Crocus* field isolated mutant with flowers lacking stamen (Tsaftaris *et al.*, in preparation).

Expressing *AGL6*-like genes under the control of the CaMV 35S promoter has dramatic effects on growth of Arabidopsis plants, such as extremely reduced plant size, very early flowering, and the formation of terminal flowers (Hsu *et al.* 2003). Even though such data, probably reflecting gain-of-function effects based on ectopic expression, are difficult to interpret, especially when obtained in a heterologous background they are repeatable after expressing *CsatAGL6a* in *Arabidopsis* under the control of CaMV 35S promoter (Tsaftaris *et al.*, in preparation). *AGL6* genes are basal genes in the MADS-box family and further work is required particularly in well studied flowers from model plants where mutant isolation in combination with transgenic and silencing technologies could better clarify their role.

CONCLUSIONS

Previous studies in dicots species, including *A. thaliana* and *A. majus*, have shown that the B-class genes *AP3/DEF* and *PI/GLO* are expressed in the developing petals and stamens throughout the ontogeny of these organs. The gene products function as heterodimers such that losses of either *AP3/DEF* or their respective partners *PI/GLO* cause homeotic replacement of petals by sepaloid structures and of stamens by carpels. Expression analysis in the different flower organs showed that unlikely to the typical model, the expression of all *CsatPI/GLO* extends into the first whorl of tepals. The extended expression of both B-class genes *paleoAP3/DEF* and *PI/GLO* to the first whorl could be supportive evidence for their heterodimerization not only to form petals or inner tepals in *Crocus*., but also in combination with the extended expression of E-class genes, for the homeotic transformation of sepals into outer whorl 1 tepals in *Crocus* (**Fig. 1B**).

Many monocot flowers have petaloid perianths in whorls 1 and 2, and it is difficult to fully account for this type of floral morphology using the classical ABC model (Coen and Meyerowitz 1991). On the basis of morphological analyses of tulip mutants, van Tunen *et al*. (1993a) hypothesized that the formation of tepals is due to the expanded expression of B-class genes into whorl 1. Moreover, a number of studies in nongrass monocots, such as tulip (Kanno *et al.* 2003), *P. equestris* (Tsai *et al.* 2004; Tsai *et al. 2005*), *A. praecox* (Nakamura *et al.* 2005), *M. armeniacum* (Nakada *et al.* 2006), and *D. crumenatum* (Xu *et al.* 2006), provide support to a simple modification of the ABC model, the so-called modified ABC model (van Tunen *et al.* 1993a). In *Crocus*, *D. crumenatum* and *M. armeniacum*, the B-class genes are expressed in whorls 1, 2, and 3, which fits the modified ABC model, but are also expressed in whorl 4, which does not fit the modified ABC model. However, the protein localization of the B-class gene products is still unclear.

In order to uncover the molecular mechanism of petaloid tepal development in monocots, such as *Crocus*, functional studies with mutant analyses and genetic transformation are needed. MADS-box gene function during the *Crocus* flower development could be revealed during studies on loss-of-function mutants such as stamenless mutant described here, where the *CsatAGL6a* gene is not expressed and moreover, during studies on combinations of such mutants in diploid *Crocus* species like *C. cartwrightianu*s, where genetic crosses between different parents are feasible.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. Yannis Patsios for his invaluable help in collecting plant material in the field and all of our lab members for their contributions, helpful comments and discussion. This work was supported by a grant from the General Secretariat for the Research and Technology (Greek Ministry of Development).

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How to reference: Author name(s) (2010) Title of chapter. In: Husaini AM (Ed) Saffron. Global Science Books, UK, pp. X-XX

Saffron Flavor: Compounds Involved, Biogenesis and Human Perception

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ABSTRACT

In recent years, saffron has increased in interest for both scientists and consumers, as it is the only spice able to give to food flavor, color and aroma to foods. In relation to flavor, picrocrocin is considered as mainly responsible for saffron's bitter taste, but other compounds structurally related to picrocrocin and flavonoids have been identified and could contribute to such a property. Further studies are necessary to establish picrocrocin sensory perception, as only its taste detection threshold has been established (10 mgL^{-1}). Even though it is well known that picrocrocin content is directly affected by the dehydration process of the spice, its generation pathway remains unclear. In this paper a comparison between the classical hypothesis and the alternative one is therefore presented. Among its flavor properties, picrocrocin is an excellent marker of saffron purity because till now its presence it is only reported in saffron. Also, it is especially useful in unmasking sophisticated adulterations carried out with pigments from *Gardenia jasminoides*, which contain the same carotenoid family as saffron. For both reasons, it is important to accurately determine picrocrocin content in order to gain the trust of saffron dealers and also for consumer satisfaction. This review summarizes the available methodologies for this proposal, giving emphasis to the gaps contained in the current ISO 3632 Standard, normally used in the international market.

Keywords: *Crocus sativus* L., picrocrocin, spice, crocetin esters, ISO/TS 3632

Symbols: $E_{1cm}^{1\%}$ is represented as $E_{1cm}{}^{1\%}$ throughout the text, figure legends and tables

CONTENTS

INTRODUCTION

In recent times, when the use of saffron in traditional medicinal recipes and beauty products has decreased, saffron is appreciated for its use in food, especially for its coloring properties. The new interest in other characteristics of this spice, such as flavor and aroma, has been enhanced as consumers become more familiar with the spice and begin to demand a higher quality of saffron. It should be noted that saffron is the only spice able to confer flavor, color and aroma in food.

The compound considered since 1930 (Lutz 1930) as mainly responsible for saffron's bitter taste is the picrocrocin, the 4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (**Fig. 1**). It is soluble in polar solvents, more soluble in water than in water-alcohol solutions (Iborra *et al.* 1992a, 1992b) and insoluble in apolar ones (Corradi and Micheli 1979a, 1979b). The structure of picrocrocin was established by Khun and Winterstein in 1934.

Picrocrocin content in saffron spice is significant, from 5% (Alonso *et al.* 2001) to 13% of dry material (Iborra *et al.* 1992b; Alonso *et al.* 2001). However, recent studies demonstrated that it could reach 18% (del Campo *et al.* 2010a) and 27% in Spanish samples (Sánchez *et al.* 2008).

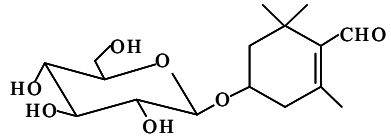


Fig. 1 Structure of picrocrocin.

Relative importance of flavor versus color properties on saffron

In international trade, saffron quality is determined by ISO 3632 standard, whose latest revision has given rise to Technical Specification ISO/TS 3632 (2003). This classifies saffron into three quality categories with regard to a large number of physical and chemical parameters such as: microscopic characteristics, presence of flower waste, mois-

ture and volatile matter content, ash content, $E_{1cm}^{1\%}$ 440 nm (coloring strength), $E_{1cm}^{1\%}$ 330 nm (related to safranal content), $E_{1cm}^{1\%}$ 257 nm (related to picrocrocin content), etc. However, only coloring strength, representing the crocetin ester content, has traditionally been of importance for companies which trade with saffron. Proof is that this Standard is not capable of discriminating between qualities with regards to volatile ($E_{1cm}^{1\%}$ 330 nm) or picrocrocin ($E_{1cm}^{1\%}$ 257 nm) content. When a sample fulfills the requirements of any category regarding coloring strength (absorbance at 440 nm), it also fulfills the other spectrophotometric parameters ($E_{1cm}^{1\%}$ 330 nm, $E_{1cm}^{1\%}$ 257 nm) for the same category. This fact has been shown by del Campo *et al.* (2010a) when 435 samples were analyzed and classified by their coloring strength.

Determination of picrocrocin content

The sample mentioned above which cannot discriminate between picrocrocin or volatile content is partially due to the current methodologies included in ISO/TS 3632 (2003) for the estimation of these compounds, which are not adequate.

Determination of these two parameters is performed through the following expression:

$$E_{1cm}^{1\%}\ \lambda = \frac{D_\lambda \times 10000}{m\,(100 - H)}$$

where λ is wavelength to which is measured the maximum of absorbance, especially at 330 nm for determination of volatile content and at 257 nm for picrocrocin, D_λ is the absorbance at about 330 or 257 nm, m is the saffron mass in the working solution expressed in grams and H is the sample moisture and volatile matter content, according to ISO/TS 3632 (2003). The determination of picrocrocin through the parameter $E_{1cm}^{1\%}$ 257 nm shows a problem of selectivity since other compounds of saffron extract, primarily crocetin esters and major compounds within the extract, have also absorbed at this wavelength (**Fig. 2**) due to the glycoside bonds, causing interferences in measurement (Tarantilis *et al.* 1994; Orfanou and Tsimidou 1996; Carmona *et al.* 2006b; Sánchez *et al.* 2008, 2009).

There is another spectrophotometric parameter ΔE_{pic}, proposed by Corradi and Micheli (1979a), for measuring the picrocrocin content, which tries to avoid the interferences of the crocetin esters when it is determined by $E_{1cm}^{1\%}$ 257 nm. ΔE_{pic} is calculated as follows:

$$\Delta E_{pic} = E_{257}^{1^0/_{000}} - E_{297}^{1^0/_{000}}$$

where the first and second parameters are the maximum extinction values at λ = 257 nm and minimum of extinction measured at λ = 297 nm for saffron aqueous extract (1:10000), respectively.

When both parameters ($E_{1cm}^{1\%}$ 257 nm and ΔE_{pic}) measured spectrophotometrically are compared with picrocrocin content measured with a more effective technique by HPLC (Sujata *et al.* 1992; Tarantilis *et al.* 1995; Lozano *et al.* 1999; Alonso *et al.* 2001), ΔE_{pic} is the most suitable as demonstrated by del Campo *et al.* (2010b). The comparison of the picrocrocin concentration overestimation obtained with $E_{1cm}^{1\%}$ 257 nm and over/underestimation using ΔE_{pic} in relation to HPLC determination for different saffron origin samples is shown throughout the coloring strength range (**Fig. 3**) (del Campo *et al.* (2010b). The samples generally presented higher values of picrocrocin content using $E_{1cm}^{1\%}$ 257 nm than the results obtained using ΔE_{pic} for all coloring strength ranges for the different countries. The higher overestimation of picrocrocin content obtained using $E_{1cm}^{1\%}$ 257 nm could be justified by the interferences of crocetin esters (Tarantilis *et al.* 1994); whereas values obtained by ΔE_{pic} gave a better estimation. The results, however, are not sufficiently reliable and not sufficiently close to the true value to accept this approximation and the possibility for using it in a company's routine quality control is not feasible.

Regardless of variables such as edaphic or climate conditions, dehydration or storage procedures, which may affect the picrocrocin content and crocetin ester, the capability of $E_{1cm}^{1\%}$ 257 nm to approximate the true values of picrocrocin is surprisingly improved when the content of crocetin esters, responsible for these interferences mainly caused by *cis* configuration, increases (**Fig. 3**). According to del Campo *et al.* (2010b) *cis*-crocetin esters are presented in lower concentration when saffron coloring strength increases and

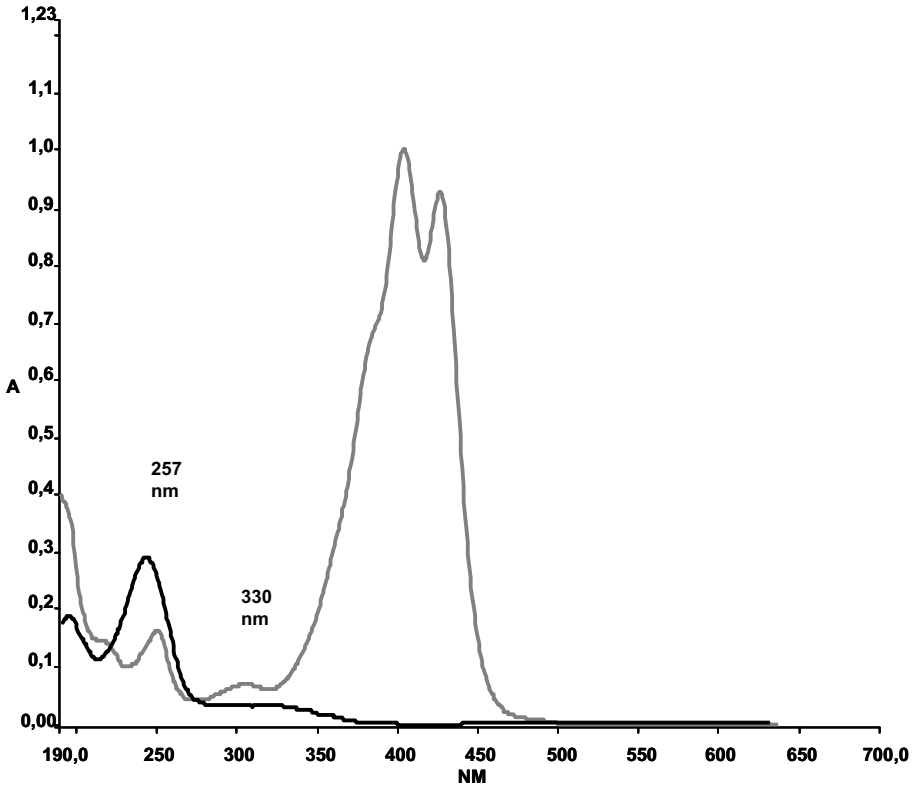


Fig. 2 UV–Vis spectra of crocetin esters and picrocrocin fractions after solid phase extraction (SPE) from a saffron aqueous extract.

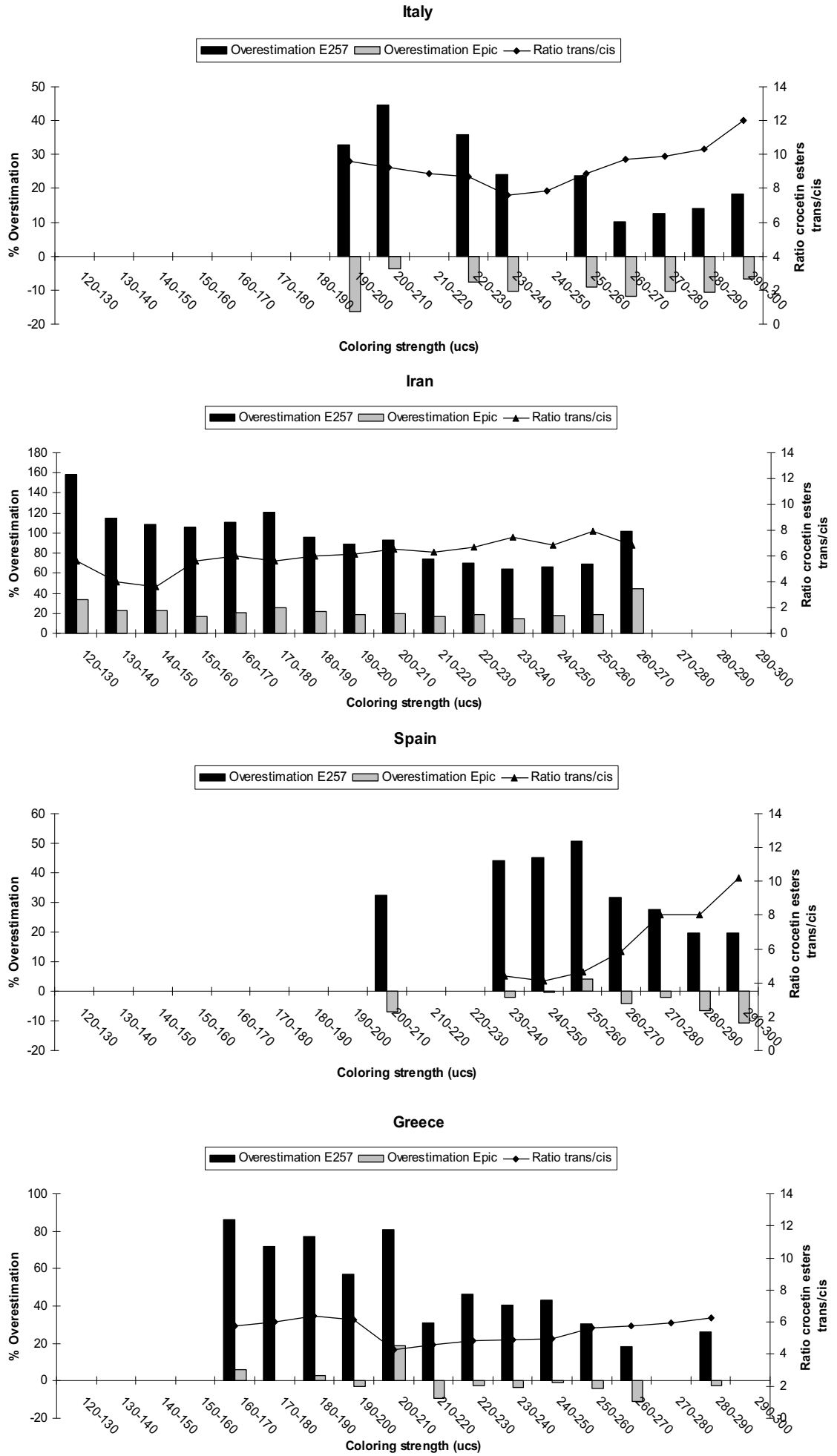


Fig. 3 Comparison of the picrocrocin content overestimation obtained with $E_{1cm}^{1\%}$ 257 nm and over/underestimation using ΔE_{pic} in each range of coloring strength and relationship with the ratio *cis*/*trans* crocetin esters for the different countries. (Adapted from del Campo *et al.* 2010b).

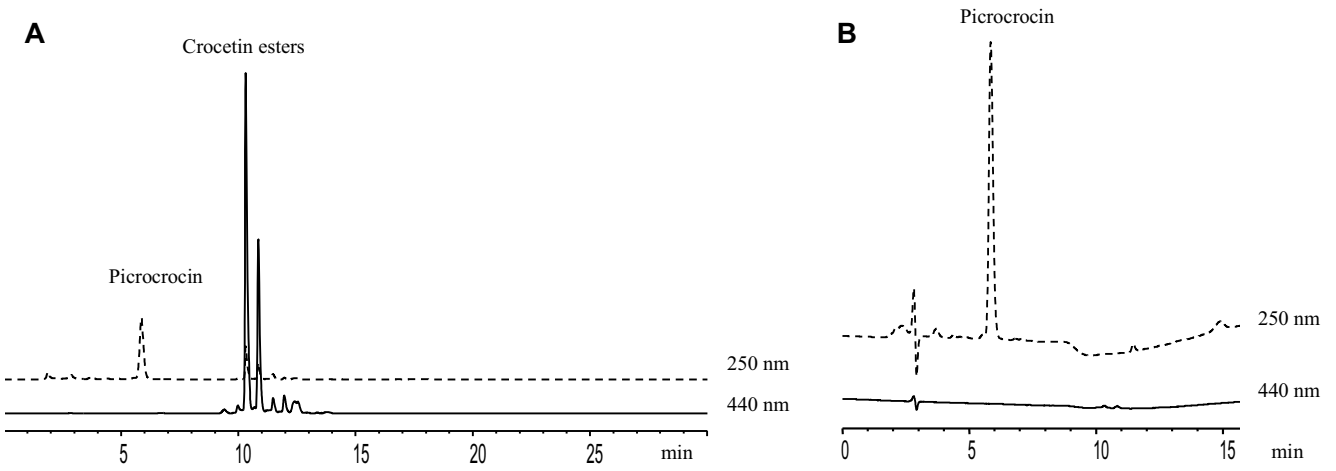


Fig. 4 Comparison of chromatograms at 250 and 440 nm of a saffron extract (A) and its corresponding picrocrocin fraction after the SPE isolation stage (B). (Adapted from Sánchez *et al.* 2009).

Table 1 Principal component statistics of NIRS calibration and validation for the $E_{1cm}^{1\%}$ 257 nm parameter measured according to ISO/TS 3632 (2003) (Adapted from Zalacain *et al.* 2005b).

Characteristic	$E_{1cm}^{1\%}$ 257 nm
Nº of analysis	221
Outliers	18
Nº independent standards	22
Nº principal components	13
SEC (Standard Error of Calibration)	6.26
SEP (Standard Error of Prediction)	6.48
R^2	0.90
Mean Value	82.27
% variance	80.37

thus the *trans/cis* ratio, observing that picrocrocin content is adjusted to real sample value.

Another technique used for determining saffron chemical composition is near-infrared (NIR) spectroscopy (Zalacain *et al.* 2005b). Principal component statistic analysis of NIRS calibration and validation for the $E_{1cm}^{1\%}$ 257 nm parameter measured according to ISO/TS 3632 (2003) were reported in **Table 1**. The number of principal components was 13, although the first two components accounted for 98% of the total variability. The low differences found between the standard error of calibration (SEC) and standard error of prediction (SEP) reveals the robustness of the equation for this parameter. For example, if the mean value of picrocrocin is 82.27, SEC value is 6.26 representing an error of about 7.6%. Although FT-NIR is a rapid technique and gives results close enough to true values, the equipment needs training and many samples are needed for a proper calibration.

But the truth is that both HPLC, and to a lesser extent NIR, require equipment that is seldom found in small or medium-size companies that process and package saffron spice. Thus, there is a real interest on the part of the companies for the development of rapid methods of routine quality control of saffron using UV–Vis spectral information. In addition, it is necessary to check if saffron is adulterated. As known, due to saffron's price, this spice was commonly adulterated with artificial colorants or *Gardenia jasminoides* extracts. The latter can increase sample coloring strength, although but picrocrocin is not present. Thus, picrocrocin is an excellent marker of saffron purity. Recently, Sánchez *et al.* (2009) has proposed a rapid method for picrocrocin routine control with a previous step of purification using a solid phase extraction (SPE) followed by UV–Vis technique. SPE is one of the most common and least expensive purification techniques and is considered as a convenient approach for sample preparation for the analysis of major and minor components of foods (Grigoriadou *et al.* 2007; Puoci *et al.* 2008). Up to the present, SPE technique had been applied to saffron extracts when detection of adulterations by artificial colorants (ISO/TS 3632, 2003; Zalacain *et al.* 2005a) was studied. The procedure for picrocrocin determination using SPE is the following: saffron aqueous extract (0.5 g L^{-1}) prepared according to the ISO/TS 3632 (2003), was centrifuged at 4000 rpm for 5 min. After having conditioned the C_{18} SPE cartridge, 1 mL of saffron extract was loaded into the SPE, washed with 10 mL water and picrocrocin was eluted with acetonitrile/water 12% (v/v) up to collecting 10 mL. The absorbance of eluted extract was measured at 250 nm in a 1 cm path length cell in the UV-Vis spectrometer. When the extract was measured by HPLC, a single peak with retention time 5.84 ± 0.03 min was detected (**Fig. 4**). The proposed method also shows a good sensitivity, the LOD is 0.30 mg L^{-1} of picrocrocin corresponding to 0.6% on a dry basis of saffron and the LOQ is 0.63 mg L^{-1} of picrocrocin that represents 1.3% on a dry basis of saffron. These values are definitely lower than 5%, minimum value for picrocrocin content (Alonso *et al.* 2001). In summary, this validated SPE gives good results for determining the content of picrocrocin in saffron spice samples from UV–Vis spectral information. The procedure is accurate, reproducible and sensitive enough for this application in samples from different countries. Furthermore, its common points with the ISO determinations in saffron, the short time necessary to carry it out and its simplicity make this procedure of particular interest for routine quality control in the industry.

Other compounds potentially involved on saffron taste

In addition to picrocrocin, other compounds that may contribute to saffron taste properties have been characterized in saffron spice although their content is much lower than picrocrocin. These compounds are structurally related to picrocrocin and flavonoids (**Fig. 5**). The identification has mainly been carried out by three research groups as shown in **Fig. 6**, with Tarantilis *et al.* (1995) proposing the first structures corresponding to 4-hydroxi-2,6,6-trimethyl-1-cyclohexen carbaldehyde 4-*O*-β-D-gentiobioside (*b*), 4-hydroxy-2,6,6-trimethyl-1-ciclohexene carboxylic 4-*O*-β-D-glucopyranoside acid (*d*) and picrocrocin (*g*). New constituents of saffron were isolated and their precursor functions with regard to their formation discussed by Straubinger *et al.* (1997, 1998a, 1998b). They identified 5-hydroxi-7,7-dimethyl-4,5,6,7-tetrahydro-3H-isobenzo-furanone 5-*O*-β-D-gentiobioside (*c*), 3′,5-dihydroxi-7,7-dimethyl perhydro-isobenzo-furanone 5-*O*-β-D-glucopyranoside (*e*) and 4-hydroxymethyl-3,5,5-trimethyl-2-ciclohexenone 4-*O*-β-D-gentiobioside (*f*). And in 2007, Carmona *et al.* (2007b) corroborated the presence of the earlier compounds such as

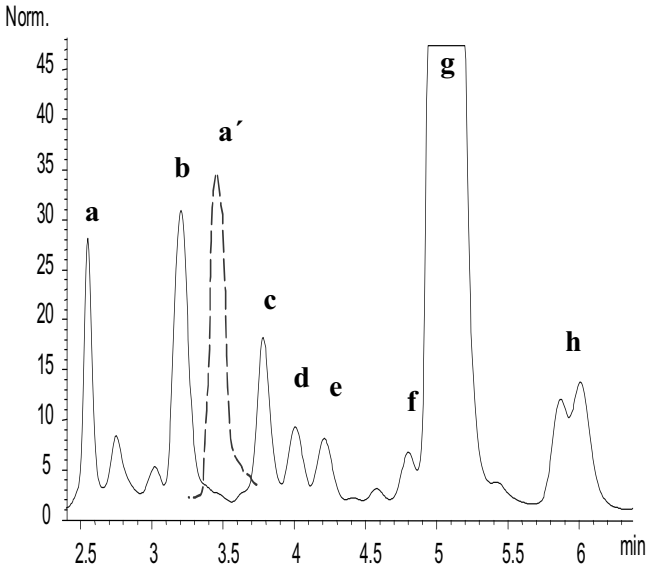


Fig. 5 Chromatogram corresponding to saffron extracts with 250 nm detection (Adapted from Carmona *et al.* 2007b).

-methyl-6-oxo-2,4 heptenate of *O*-β-D-gentiobioside (*a*) (**Fig. 5**) and extended the number of compounds tentatively identified.

After chromatographic separation it is easy to identify picrocrocin (*g*) since it is the most important substance with absorption at 250 nm, but other compounds appear with it in an aqueous saffron extract. Some of them have UV-Vis spectra almost identical to picrocrocin, as can be seen by the structure assigned to each of them and their mass spectra as reported in **Fig. 6**. The 2-methyl-6-oxo-2,4 heptenate of *O*-β-D-gentiobioside structure was assigned to peak *a* in **Fig. 5**. This compound was previously identified in saffron by the Winterhalter group (Straubinger *et al.* 1997, 1998a, 1998b). Signals at m/z 501 and 367 correspond respectively to $[M+Na]^+$ and the loss of the linear chain $[M-C_7OH_{10}]^+$ from the molecule. In chromatographic mobile phases when formic acid was added in order to promote ionisation of the substances, a considerable decrease was observed in the presence of this compound, besides the apparition of other compounds with an approximate 3.5 min retention time, shown by discontinuous line. It might be the same compound that had lost a glucose and remained in ionic form (m/z 361 $[M+2Na]^+$ in positive ion mode and at m/z 337 $[M-H+Na]^-$ in negative ion mode). Peak *b* was identified as 4-hydroxi-2,6,6-trimethyl-1-ciclohexen carbaldehyde 4-*O*-β-D-gentiobioside, where signals at m/z 515 and 339 correspond to $[M+Na]^+$ and the loss of the ring $[M-C_{10}OH_{16}+H]^+$. Peak *c* was identified as 5-hydroxy-7,7-dimethyl-4,5,6,7-tetrahydro-3H-isobenzo-furanone-5-*O*-β-D- gen -tiobioside after assigning their corresponding ions $[M+Na]^+$, $[M\text{-glucose}+2H+Na]^+$ and $[M\text{-glucose} + Na]^+$ to signals at m/z 527, 369 and 185. The signal at m/z 369 reflects the opening of lactone at the same time as one of the glucose molecules is lost. Straubinger *et al.* (1998b) found the same compound, but less glycosylated. Instead of containing the gentiobiose molecule, they only found a glucose molecule. The compound assigned to peak *d* coincides with what was shown by the Tarantilis and Winterhalter groups as 4-hydroxi-2,6,6-trimethyl-1-ciclohexene carboxylic 4-*O*-β-D-glucopyranoside acid. Fragmentation signals at m/z 369 and m/z 167 correspond to $[M+Na]^+$ and $[M\text{-glucose-}O+Na]^+$, respectively. Fragmentation signals for peak *e* were coherent with a compound of $C_{10}O_4H_{14}$ molecular form besides the glucose molecule. After thoroughly studying signals at m/z 383 and 367 corresponding to $[M-Na]^+$ and $[M-OH+Na]^+$, this peak was identified as 3´,5-dihydroxi-7,7-dimethyl perhydro-isobenzo-furanone 5-*O*-β-D-glucopyranoside, the hydrated form of the compound identified in peak c but with one less glucose. Peak *f* whose signals were at m/z 515 $[M+Na]^+$ and m/z 357 $[M-C_9OH_{12}]^+$, which assumes the freedom of the CH_3O-Gen fragment, was assigned to the 4-hydroxymethyl-3,5,5-trimethyl-2-cyclohexenone 4-*O*-β-D-gentiobioside compound. The remaining fragmentation signals (303, 232, 124) can be justified by different breakdowns of sugar. Signals from the majority peak *g* perfectly confirm that it is picrocrocin: m/z 353 corresponds to $[M+Na]^+$, while m/z 185 reflects the loss of glucose $[M\text{-glucose}+Na]^+$. Lastly, peak *h*, consisting of two shoulders, was granted the structure of two isomers, the 4-hydroxy-3,5,5-trimethyl-2-ciclohexenone 4-*O*-β-D-glucopyranoside (m/z 337 $[M+Na]^+$), compounds identified by the Straubinguer *et al.* (1998b).

On the other hand, the first identification of a flavonoid in saffron spice (by mass spectrometry) was made by Tarantilis *et al.* (1995), proposing a kaempferol structure with a disaccharide moiety (**Fig. 7A**). Straubinger *et al.* (1997) identified kaempferol 7-*O*-glucopyranosyl-3-*O*-sophoroside and kaempferol 7-*O*-sophoroside (**Fig. 7B**) by NMR and MS after counter-current preparative chromatography. Taking this determination into account, the same authors considered that the identification of a new flavonoid named kaempferol 3-*O*-gentiobioside carried out by Lozano *et al.* (1999) was not correct (Winterhalter and Straubinger 2000). Moreover, other flavonoids may be found in saffron spice, as they have already been described in other *Crocus* species (Nørbæk and Kondo 1999). The last study on the profile of flavonoids in saffron (Carmona *et al.* 2007b) showed that in aqueous extract only flavonol compounds of the flavonoid family were found. After acid hydrolysis, all the flavonoids gave kaempferol as an aglycone. They were kaempferol derivatives with three and two hexoses (**Fig. 7**). Their fragmentation patterns coincided with two standards, the -3-sophoroside-7-glucoside and the -3-sophoroside of kaempferol, respectively (Ferreres *et al.* 2004). All these data confirm the structures reported previously by Straubinger *et al.* (1997) for the main saffron flavonoids.

It remains to establish what their contribution to the saffron taste could be, as it is well known that flavonoids have many functions in the biochemistry, physiology and ecology of plants, as well as in both human and animal nutrition (Forkmann and Martens 2001).

Biogenesis of compounds responsible for flavor and bioconversion

The classical biogenesis pathway for the generation of picrocrocin and subsequent volatile compounds is shown in **Fig. 8**. Zeaxanthin breaks on both ends to generate crocetin-dialdehyde plus two molecules of picrocrocin which is later converted into safranal, while crocetindialdehyde is oxidized to give rise to crocetin and later on crocetin esters by the action of a glycosyltransferase (Côté *et al.* 2000).

This hypothesis was supported by Buchecker and Eugster (1973) who confirmed that the configuration of the carbon supporting the hydroxyl group in picrocrocin and in the zeaxanthin, the R configuration, is the same (**Fig. 9**). Also, Himeno and Sano (1987), followed by Lozano *et al.* (1999), proposed that during the dehydration process, 4-hydroxy-2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde (HTCC) could be the intermediate compound for the synthesis of safranal from picrocrocin either because of the temperature reached or the action of glycosidases (**Fig. 10**).

There is some evidence that the three types of compounds: crocetin esters, picrocrocin (and its related compounds) and volatiles could be interrelated in a different manner, as crocetin esters can generate safranal and other volatile compounds as much as picrocrocin and their analogues (Carmona *et al.* 2007a). This evidence was shown when the effects of dehydration processes on the different compounds in saffron spice were studied.

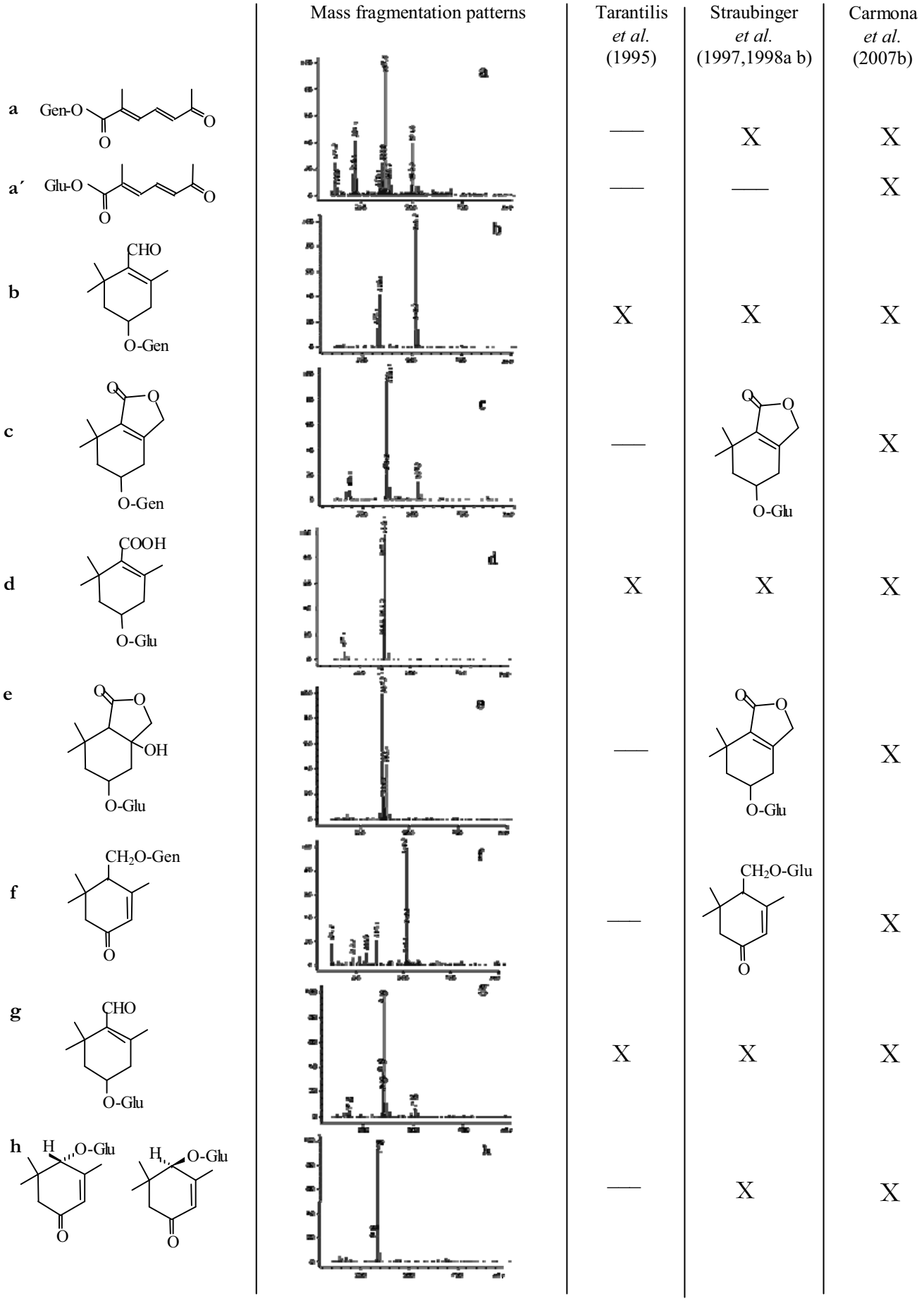


Fig. 6 Structures and mass fragmentation patterns proposed for the compounds in Fig. 5 detected by HPLC-DAD at 250 nm in aqueous saffron extract. In columns at the right, results are compared to those previously identified by other authors: Tarantilis *et al.* (1995), Straubinger *et al.* (1997, 1998a, 1998b) and Carmona *et al.* (2007b). Note: X means that these authors had already proposed its existence. When the compound is not the same but a similar one, its structure is given.

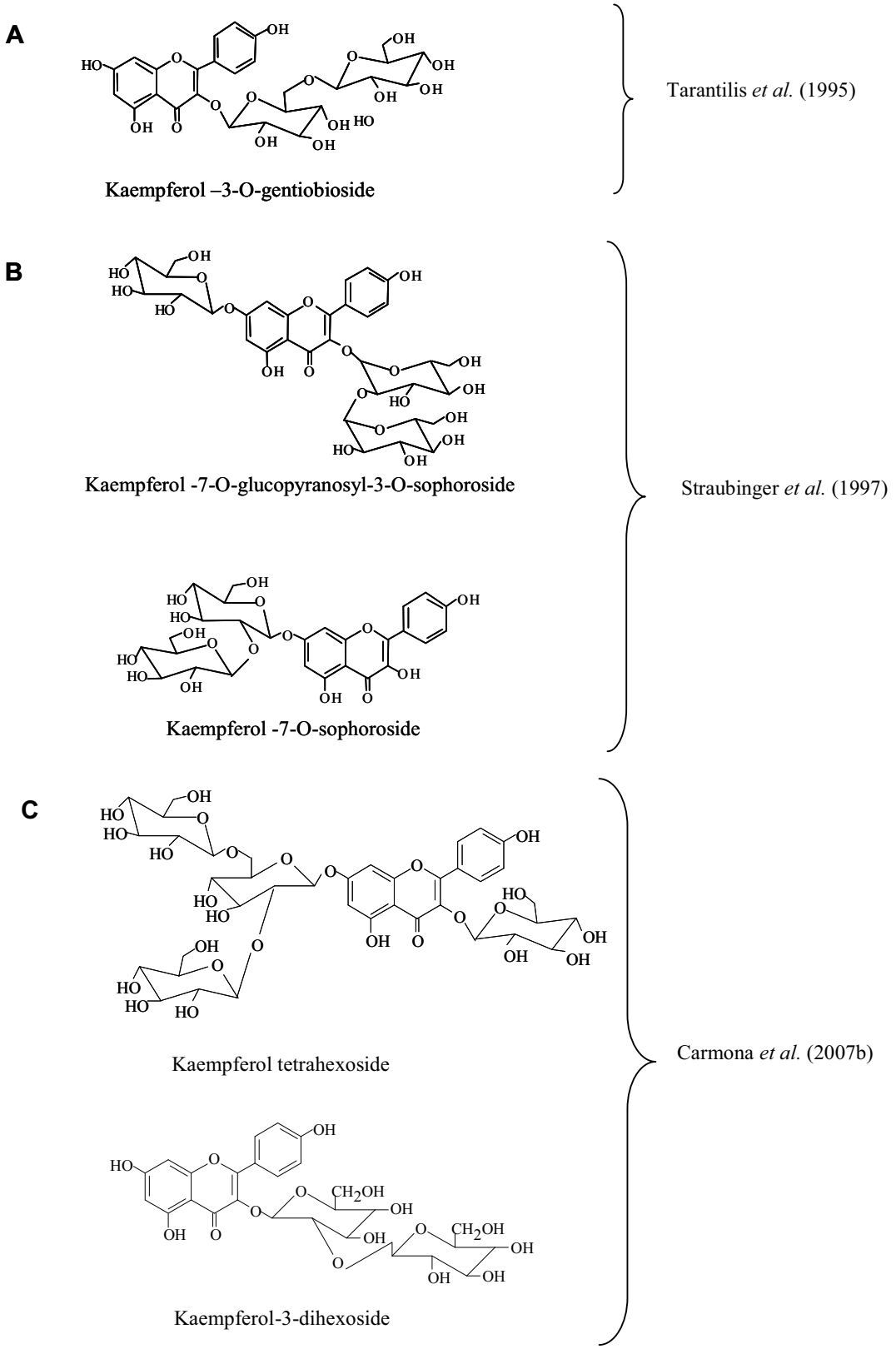


Fig. 7 Structure of the flavonoids identified in saffron spice.

1. Effect of post-harvesting treatments on picrocrocin

While it is known that the dehydration procedure is responsible for saffron sensorial properties: color, flavor and aroma (Pardo *et al.* 2002; Carmona *et al.* 2006a, 2006c), the best conditions to carry out this postharvest treatment remain unclear. Changes in the compounds responsible for these characteristics have been studied when dehydration processes are performed at low (Nauriyal *et al.* 1997; Hassnais 1998; Ati-Oubahou and El-Otomani 1999), high (Carmona *et al.* 2005; Gregory *et al.* 2005) and at mild temperatures (Tammaro 1999; Ordoudi and Tsimidou 2004; del Campo *et al.* 2010a).

In this last paper, the effect of different mild conditions applied to saffron samples with the same origin was investigated. The results showed that picrocrocin content is higher when the higher temperature is applied within the range assayed (between 18 and 55°C). According to Alonso *et al.* 1993 when similar temperatures were used (25-40°C), the same behavior was observed for picrocrocin. The increment

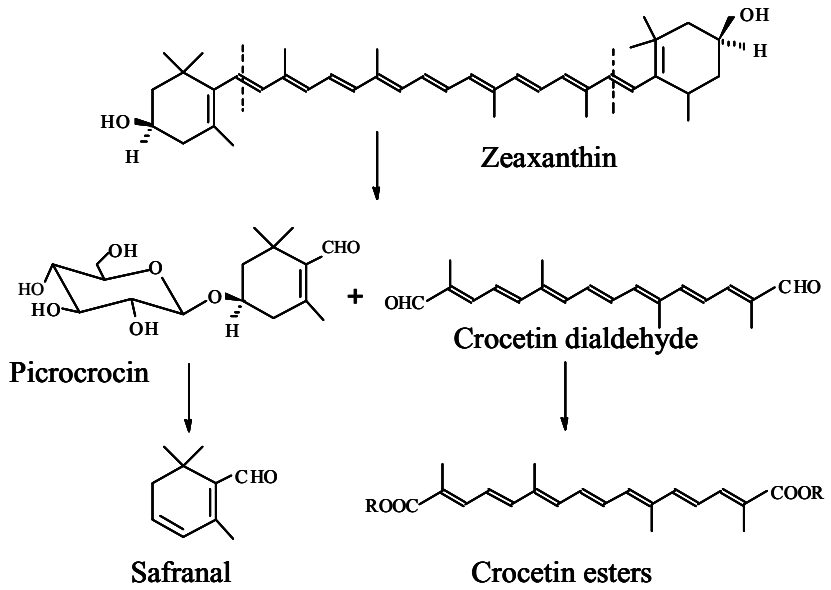


Fig. 8 Classical biogenesis pathway for the generation of picrocrocin and volatile compounds in saffron. (Adapted from Carmona *et al.* 2006d).

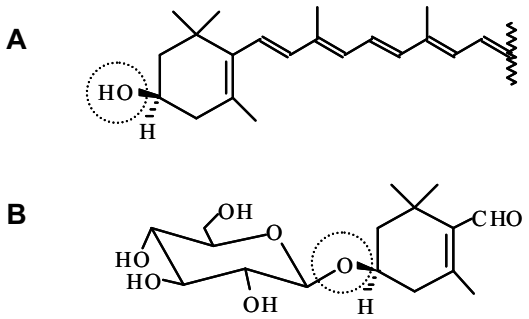


Fig. 9 Diagram representing the configuration of the carbon that supports the hydroxyl group in zeaxanthin (A) and picrocrocin (B).

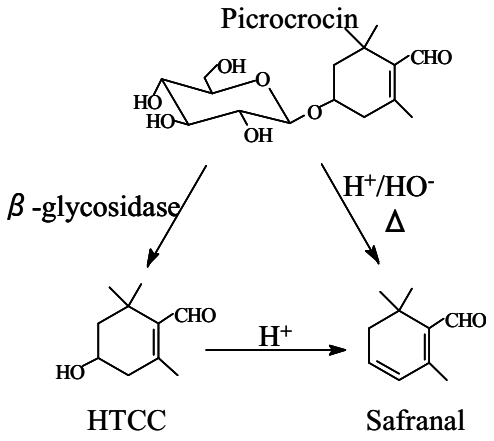


Fig. 10 Diagram of the chemical or enzymatic formation of safranal from picrocrocin. (Based on Himeno and Sano 1987).

of picrocrocin content with the dehydration temperature has been previously described for higher temperatures used in the Spanish dehydration process (Carmona *et al.* 2005), revealing that picrocrocin content increases up to 70°C and then decreases. It is worthy to mention that this outcome is in disagreement with the accepted theory that during the dehydration procedure, picrocrocin, the supposed precursor of safranal and other volatiles, is transformed in order to generate these compounds, thus reducing its content.

There is some evidence that high temperature procedures would promote the production of compounds responsible for flavor and aroma. Results from different authors employing drying conditions of 80°C for 30 min in an oven (Loskutov *et al.* 2000), presumably without air circulation, 110°C for 2 min (Pardo *et al.* 2002; Carmona *et al.* 2006b) and 70°C for 30 min with a strong air flow (Loskutov *et al.* 2000) support the idea that during the dehydration stage, important physical and chemical changes take place that are not explained. The new hypothesis proposed by Carmona *et al.* (2006d) for the generation of the compounds responsible for flavor and aroma of saffron is presented in **Fig. 11**. Aroma formation would take place from crocetin esters by the action of a carotenase. In this way it is easy to explain why odorous C9 and C10 compounds are found in one plant and not in gardenia. The presence of an enzyme could be responsible for the difference. This hypothesis is compatible with existing knowledge on the formation of six-member rings in carotenoid linear ends. Besides being simple, it complies with the principle of metabolic economy, a single route that can progress forward when given additional enzymes. This hypothesis is coherent with the disappearance of crocetin esters found by Himeno and Sano during anthesis (1987) and with the other relevant results found. During stigma development, crocetin esters and picrocrocin would maintain a more or less constant proportion (Himeno and Sano 1987) since HTCC, found in very small amounts at this stage, would be glycosylated to picrocrocin. This mechanism, customarily used by plants to accumulate metabolites in large quantities to avoid degradation or cell damage, would prevent their oxidation. At this moment the safranal content is negligible (**Fig. 12A**).

At the time of anthesis and during the long dehydration procedures at low temperature, specific or unspecific glycosydases would act on picrocrocin (Lozano *et al.* 1999), changing it again into HTCC. Its concentration would increase greatly, not only through the transformation of picrocrocin, but also because carotenase would actively work on crocetin esters (**Fig. 12B**). Raina *et al.* (1996) emphasized that temperatures lower than 35-45°C required too long a drying period, resulting in excessive enzymatic degradation of crocetin esters.

When non enzymatic cleavage is possible for the low water activity after dehydration or due to the high temperature reached during the dehydration procedure, the crocetin esters, more labile compounds than picrocrocin, could convert directly into HTCC and safranal without passing through picrocrocin generation (**Fig. 12C**), permitting at the same time an increment in picrocrocin content (Loskutov *et al.* 2000; Pardo *et al.* 2002).

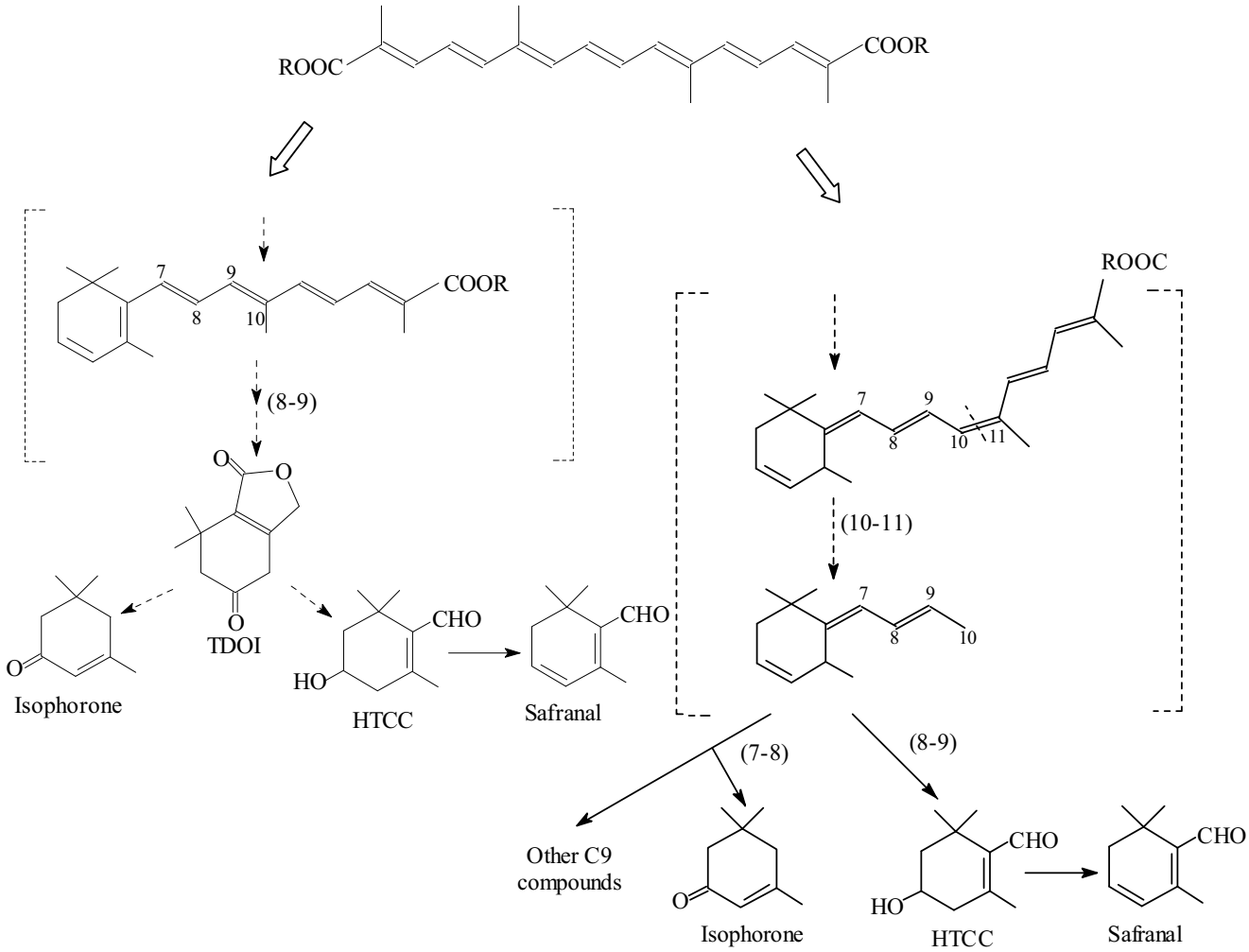
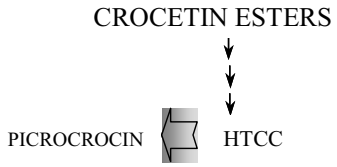
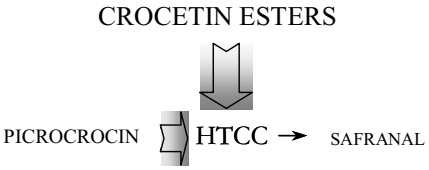


Fig. 11 New hypothesis for aroma formation from *Crocus sativus* L. crocetin esters. (Adapted from Carmona *et al.* 2006d).

(A) **Before anthesis**



(B) **During anthesis and long dehydration procedure at low temperature**



(C) **After dehydration at any temperature and during dehydration at high temperature**

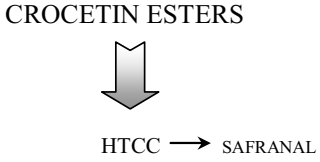


Fig. 12 Current hypothesis proposed for picrocrocin generation and conversion at different saffron stages. (Adapted from Carmona *et al.* 2006d).

Sensory perception

Only a few studies deal with saffron taste (Sarma *et al.* 1991; Narashimhan *et al.* 1992; Raina *et al.* 1996; Pardo *et al.* 2002) and, in particular, with picrocrocin and bitter taste. Sarma *et al.* (1991) made the first report on the sensory analysis of saffron produced through tissue cultures of *Crocus sativus*. In this study, stigma-like structures were produced in tissue cultures (TC stigmas) from the ovary explants of *C. sativus* on MS medium. Crocin and picrocrocin were found to be 6 to 11 times lower in TC stigmas than in the natural stigmas. The saffron obtained from tissue cultures was subjected to sensory analysis and compared with the data obtained from chemical analysis. The sensory data indicated that the saffron pigments produced in tissue cultures were one tenth those of natural stigmas. Sensory profile tests showed that the tissue culture saffron was low in floral, spicy and fatty characteristics as compared to saffron obtained from flowers.

Narasimhan *et al.* (1992) reported the assessment of the recognition threshold concentration of flavor components, the intensity of flavor attributes and the establishment of the dose-response relationships over a wide range of stimulus concentration. In this paper, the mean scores of four different saffron samples were provided. Samples A (the select grade) and B (lower grade) came from Kashmir, having a flavor threshold of 1.25 and 1.67 mg 100 mL^{-1}, respectively. Sample C, a commercial saffron, had a threshold of 2.50 mg 100 mL^{-1}, whereas for sample D, a saffron grown *in vitro*, it was 20 mg 100 mL^{-1}. Thus, the threshold of saffron flavor proved an important indicator of its strength (concentration), as well as the maturity of the sample or the part of the plant.

In addition, Raina *et al.* (1996) studied the effect of post-harvest processing on the flavor of saffron, and observed that a prolonged storage time affected the pigments and flavor concentration to a great extent, but proper storage (with 5% moisture) and packaging in a polystyrene box wrapped with MXXT cellophane reduced saffron deterioration and increased the shelf-life of the product.

In the same field are the sensory analyses carried out by Pardo *et al.* (2002), who developed affective (102 consumer judges) and discriminatory (15 expert judges) tests to determine the effect on the human perception of saffron samples dehydrated in different ways. The results (**Table 2**) showed that, as regards color, consumer judges preferred the samples dehydrated with hot-air (DHA) that were brightest and showed significant difference between toasted and dehydrated samples at room temperature (DRT). With respect to the sensory aroma parameters, consumer judges preferred DHA and toasted samples. As for the flavor parameters, significant differences between the samples were not found in the affective judges, whereas the expert judges found DHA and toasted samples more bitter than DRT ones. In all cases, saffron dehydrated at room temperature was the worst rated as it had less color, flavor and aroma.

Recently, Sánchez *et al.* (2009) carried out a study on sensory analysis of picrocrocin, previously isolated and purified (96%), to evaluate saffron's bitterness. In this study, the taste detection threshold of picrocrocin in aqueous extract was determined according to ISO 4120 (2004). The picrocrocin taste detection threshold was set at 10 mg L^{-1}. Although further research on saffron bitterness is necessary, the taste detection threshold of picrocrocin adds crucial information to take advantage of saffron's taste potential and optimize its usage in food.

Table 2 Sum of the scores given for the sensory parameters evaluated by a discriminatory test (Adapted from Pardo *et al.* 2002).

Color		Aroma	Bitterness
Red tone	Brightness		
15a	17a	26a	18a
31a	28a	59c	50b
60b	57b	43b	58b
59b	56b	36b	47b
60b	67c	61c	52b

DRT: dehydrated at room temperature; DHA: dehydrated with hot-air.
Values followed by different letters within a column are significantly different at $p<0.05$ (Duncan´s test).

CONCLUSION

Knowledge of picrocrocin is gradually becoming more important in the international trade of this spice. On one hand, consumers begin to demand a more broadly-focused saffron quality which is not based solely on color. This consumer demand has led to more accurate methods to determine picrocrocin content in an easy and reliable way. These methods have established that picrocrocin content is directly related to the handling of the spice, both during the dehydration process and storage time. From these studies, it was learned that it is much more resistant to heat treatment and degradation over time than at first thought. For this reason, it is quite possible that picrocrocin is not the only or the major precursor of saffron volatile generation, as was thought until now.

Picrocrocin is an excellent marker of saffron purity against its sophisticated adulteration when *Gardenia jasminoides* is used to increase its color, since picrocrocin is present only in the saffron spice.

ACKNOWLEDGEMENTS

We thank the Consejería de Educación y Ciencia of the JCCM and the European Social Fund for funding this work with the Exp. 09/09-C; the Ministerio de Educación y Ciencia and FEDER (CE) for the AGL2007-64092/ALI project.

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How to reference: Author name(s) (2010) Title of chapter. In: Husaini AM (Ed) Saffron. Global Science Books, UK, pp. X-XX

Understanding Carotenoid Metabolism in Saffron Stigmas: Unravelling Aroma and Colour Formation

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ABSTRACT

Unusual in plants, the *Crocus sativus* stigma accumulates large amounts of specific glucosylated apocarotenoids that contribute to the colour, flavour and aroma of saffron spice, the processed stigma of this species. These compounds are generated from the oxidative cleavage of carotenoids followed by specific glucosylation steps. Apocarotenoid biosynthesis and its regulation during saffron stigma development is a complex process that occurs alongside the development of the stigma, changing the organoleptic properties of the spice obtained. The expression pattern of the genes involved in the production of these compounds, their precursor's changes as the stigma develops and the control of gene expression are all thought to be the main regulatory mechanisms for alterations in apocarotenoid levels. In *C. sativus* the carotenoid cleavage enzymes are especially important due to their involvement in apocarotenoid formation. Although several of these enzymes have been recently characterized, the enzyme involved in the generation of the main saffron apocarotenoids remains at the moment elusive. This brief review provides a comprehensive picture of the molecular regulation of colour and flavour biosynthesis in *C. sativus* along with what is currently known about the players involved.

Keywords: apocarotenoids, chromoplast, *Crocus sativus*, gene duplication, gene expression
Abbreviations: **ABA**, abscisic acid; **CCD**, carotenoid cleavage dioxygenase; **CHY**, carotene hydroxylase; **GGPP**, geranylgeranyl diphosphate; **GTs**, glycosyltransferases; **HMGR**, 3-hydroxy-3-methylglutaryl CoA reductase; **IPP**, isopentenyl diphosphate; **LCY**, lycopene cyclase; **MEP**, methyl-erythritol phosphate; **MVA**, mevalonate pathway; **NCED**, 9-*cis*-epoxycarotenoid dioxygenase; **PDS**, phytoene desaturase; **PSY**, phytoene synthase; **VDE**, violaxanthin de-epoxidase; **ZEP**, zeaxanthin epoxidase; **ZDS**, zeta-carotene desaturase

CONTENTS

BIOLOGICAL IMPORTANCE OF CAROTENOIDS AND APOCAROTENOIDS

Plant carotenoids are C_{40} carbohydrates with a chain of conjugated double bonds, creating a chromophore that absorbs light in the blue range of the spectrum. Flowers and fruits of many species are coloured due to the accumulation in the chromoplasts of carotenoid pigments that provide distinct colouring to the tissues (Hirschberg 2001). These colours, ranging from yellow to orange and red, visually attract pollinators and facilitate seed dispersal by animals. The carotenoids in flowers and fruits are considered sec-ondary metabolites as they contribute to plant fitness but are not necessarily essential for physiology in these tissues. On the other hand, in photosynthetic organisms, carotenoids have important roles as accessory light-harvesting pigments, effectively extending the range of light absorbed by the photosynthetic apparatus. They also perform an essential photoprotective role by quenching triplet state chlorophyll molecules and scavenging singlet oxygen and other toxic oxygen species formed within the chloroplast (Niyogi 2000). Heat and light stress tolerance is also mediated by carotenoid antioxidants that protect membranes from lipid peroxidation (Davison 2002; Havaux *et al*. 2007; Johnson *et al*. 2007). Constitutive expression of carotenoid biosynthesis genes has been observed in all green tissues examined. By contrast, accumulation of high concentrations of carotenoids in flowers and fruits is correlated with upregulation of genes that enhance the flux of the biosynthetic pathway (Botella-Pavia and Rodríguez-Concepción 2006).

Apart from these functions, carotenoids serve as precursors of several physiologically important compounds, synthesized through oxidative cleavage and generally known as apocarotenoids (Walhberg and Eklund 1998). Representative examples are the ubiquitous chromophore retinal (von Liting and Vogt 2000; Redmond *et al*. 2001), chordate morphogen retinoic acid (Campo-Paysaa *et al*. 2008), phytohormone abscisic acid (Schwartz *et al*. 1997) and fungal phero-

Fig. 1 Crocetin esters are present in *Crocus sativus* stigmas. *C. sativus* is a small bulbous plant characterized by its long red stigmas.

mone trisporic acid (Burmester *et al*. 2007). In addition, a group of C_{15}-apocarotenoids, the strigolactones, are essential signalling molecules which attract both symbiotic arbuscular mycorrhizal fungi and parasitic plants (Akiyama 2007; Bouwmeester *et al*. 2007). It was recently shown that strigolactone functions as a novel plant hormone regulating shoot branching (Gomez-Roldan *et al*. 2008; Umehara *et al*. 2008). Furthermore, the development of arbuscular mycorrhiza is accompanied by accumulation of cyclohexenone (C_{13}) and mycorradicin (C_{14}) derivatives (Schliemann *et al*. 2008), apocarotenoids arising from the cleavage of xanthophylls and leading to yellow pigmentation of the roots (Walter *et al*. 2000). C_{13}-apocarotenoids, such as β-ionone, constitute an essential aroma note in tea, grapes, roses, tobacco and wine (Rodríguez-Bustamante and Sánchez 2007). These volatile compounds are also synthesized and released by cyanobacteria (Jüttner 1984), and have important ecological roles as sensory signals. Some apocarotenoids, such as bixin and crocetin, represent plant pigments of economic value. Crocetin is synthesised by *C. sativus* (**Fig. 1**) and other related species, *Buddleja* (Liao *et al*. 1999), *Jacquinia angustifolia* (Eugster *et al*. 1969), *Coleus forskolii* (Tandon *et al*. 1979), *Gardenia jasminoides* (Pfister *et al*. 1996), and by the cyanobacterium *Microcystis aeruginosa* (Jütner and Höflacher 1985), but in none of these species does crocetin accumulate at levels as high as those detected in saffron stigmas.

Carotenoids are important not only for plants, but also for animals and humans since they have long been recognized as essential nutrients and beneficial health compounds (Fraser and Bramley 2004). As animals and humans are unable to synthesize carotenoids *de novo*, they have to depend on diet for these essential products. Xanthophylls, such as lutein and zeaxanthin, are essential components of the macular pigments in eyes and offer protection against macular degeneration, the leading cause of age-related blindness (Krinsky *et al*. 2003). "Pro-vitamin A" carotenoids, such as β-carotene and α-carotene, provide the primary dietary sources of vitamin A. Some carotenoids such as lycopene, rich in the tomato, are strong antioxidants and have a protective function in reducing the risk of cancer and cardiovascular diseases (Chan *et al*. 2009). All these important health benefits to animals and humans make the research on carotenoid metabolism exceptionally important.

CAROTENOID BIOSYNTHESIS IN FLOWERING PLANTS

In higher plants, carotenoids are derived from isopentenyl diphosphate (IPP) and are produced in plastids by the methyl-erythritol phosphate (MEP) pathway (**Fig. 2**) (Lichtenthaler 1999; Hunter 2007). Genetic and molecular studies have established that nuclear genes encode all the enzymes of the pathway (reviewed in Cunningham and Gantt 1998; Botella-Pavia and Rodriguez-Concepcion 2006; Giuliano *et al*. 2008; Lu and Li 2008). Four molecules of IPP are converted to geranylgeranyl diphosphate (GGPP) (C_{20}) by the action of IPP isomerase (IPI) and GGPP synthase (GGPS). The condensation of two molecules of GGPP by phytoene synthase (PSY) gives rise to 15-*cis*-phytoene (C_{40}), the first specific compound in the carotenoid pathway (**Fig. 3**). Phytoene is converted into lycopene by the action of two desaturases: phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS). This pathway gives rise to poly-*cis* compounds that are converted to their all-*trans* forms through the action of the carotenoid isomerases CrtISO (Isaacson *et al*. 2002; Park *et al*. 2002) and ZISO (for 15-*cis* zeta-carotene isomerase) (Li *et al*. 2007). Lycopene is the substrate of two competing cyclases: epsilon-cyclase (LCY-ε) and beta-cyclase (LCY-β), acting together on the two ends of the molecule and leading to the formation of α-carotene, whereas the action of LCY-β alone forms β-carotene. Beta- and α-carotene are hydro-xylated by non-heme (CHY1, CHY2) as well as cyto-chrome P450 (CYP97A and CYP97C) hydroxylases. CYP97C hydroxylates the epsilon-ring of lutein (Tian *et al*. 2004). Beta-xanthophylls are epoxidated–de-epoxidated by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), giving rise to the xanthophyll cycle. The subsequent opening of the cyclohexenyl 5-6-epoxide ring in violaxanthin gives rise to neoxanthin. A hypothetical enzyme complex containing enzymes from the isoprenoid pathway, IPI and GGPS, membrane-associated enzymes of the carotenoid pathway, PDS, PSY, ZDS, CrtISO, ZISO, and LCY, have long been hypothesized (Cunningham and Gantt 1998).

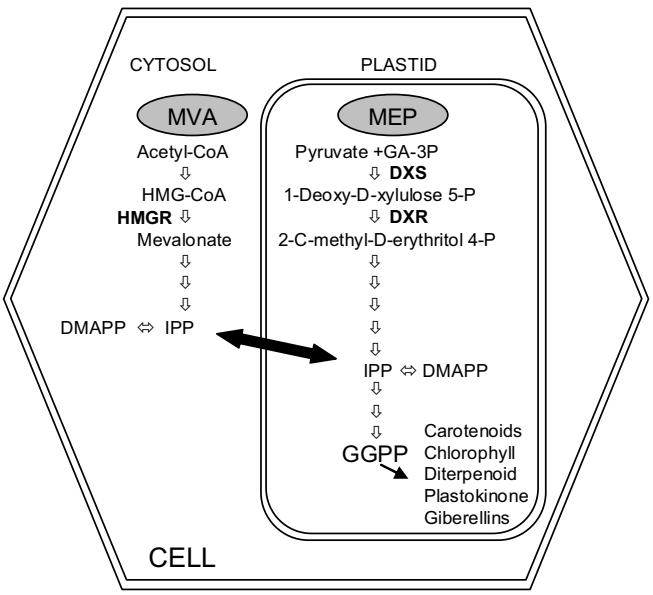

Fig. 2 Location and expression of the selected candidate genes and products in the isoprenoid pathway. A mevalonic acid (MVA) pathway is localized in the cytosol and endoplasmic reticulum to supply IPP for the synthesis of cytosolic and mitochondrial isoprenoids. The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway is localized in plastids. In all three compartments, IPP (C5) is utilized by prenyltransferases to produce a variety of linear allylic prenyl diphosphates of increasing size. Geranyl diphosphate (C10), farnesyl diphosphate (C15) and geranylgeranyl diphosphate (C20) are key intermediates for the synthesis of the wide range of end products derived from the isoprenoid pathway.

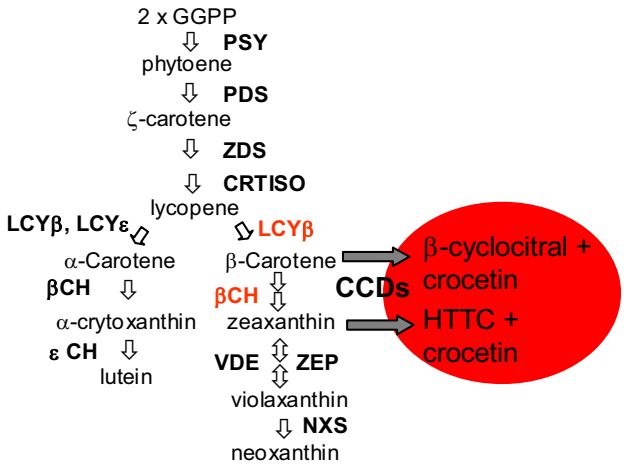

Fig. 3 Schematic carotenoid biosynthetic pathway in *C. sativus* stigma and main apocarotenoids generated. Enzymatic reactions are represented by arrows. GGPP, geranyl geranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, carotene desaturase; CRTISO, carotene isomerase; LCYB, lycopene B-cyclase; LCYε, lycopene E-cyclase; BCH, B-carotene hydroxylase; ECH, E-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NXS, neoxanthin synthase; CCD, carotenoid cleavage dioxygenase; HTTC, 2,6,6-trimethyl-4-hydroxy-1-carboxaldehyde-1-cyclohexene. In red text are the key enzymes that control carotenoid biosynthesis in saffron stigma.

CHROMOPLAST-SPECIFIC CAROTENOID BIOSYNTHESIS PATHWAY

The amounts and identities of the various carotenoids in the photosynthetic membranes of green plants are relatively well conserved. In contrast, carotenoid pigmentation in non-green plant plastids varies broadly both in quantity and composition. In flowers, fruits and roots, carotenoids are synthesized and also located in chromoplasts, with organelles being related to and often derived from these chloroplasts. There is also great diversity in the morphology of chromoplasts (Sitte *et al*. 1980). Simultaneous with chromoplast formation, an active synthesis of carotenoids begins and special carotenoid-bearing structures start to develop. These are structurally diverse, and several types are often present in the same organelle. Some carotenoid-containing structures may be transient and disappear again in senescent chromoplasts. The most common carotenoid-containing structures present in senescent chromoplasts are plastoglobules, spherical lipid droplets that lie singly or in groups in the chromoplast stroma (Brehelin and Kessler 2008). Furthermore, in plastoglobules from red pepper chromoplasts, Ytterberg *et al*. (2006) identified the carotenogenic enzymes ZDS, LYC-β or CYC-β and two β-carotene hydroxylases.

Recent studies suggest the presence of a specific chromoplast pathway for carotenoid biosynthesis in order to enhance carotenogenesis in flowers and to increase pigmentation in fruit chromoplasts. This pathway is generated by gene duplication of specific carotenogenic genes (Gallagher *et al*. 2004). Genes coding for GGPS, PSY, LYC, and CHY have been isolated from different plants, and each is encoded by at least two genes. In each of these enzymes, one isoform is constitutively expressed in leaves, whereas the other is specific for chromoplasts in flowers and/or fruits (Galpaz *et al*. 2006). In *C. sativus* two genes for PSY (Rubio *et al*. 2003), CHY (Castillo *et al*. 2005) and LCY (Ahrazem *et al*. 2009) have been identified. The presence of a specific chromoplast pathway of carotenoid biosynthesis in *C. sativus* might play an important role in the high apocarotenoid accumulation in stigma tissue.

Transcriptional regulation of the chromoplast-specific carotenoid gene expression appears to be a major mechanism regulating the biosynthesis and accumulation of specific carotenoids. Examples are found in tomato and pepper fruits and flowers, where the accumulation of specific carotenoids coincides with increased expression of upstream carotenogenic genes and reduced expression of genes downstream from the accumulating carotenoids (Hirschberg 2001). Post-transcriptional regulation at enzymatic levels also plays a role in controlling carotenoid biosynthesis and accumulation. Metabolic turnover of carotenoids by carotenoid cleavage dioxygenases helps to maintain the steady levels of carotenoids, and also produces important apocarotenoid molecules.

PLANT CAROTENOID CLEAVAGE OXYGENASES AND THEIR APOCAROTENOID PRODUCTS

The first gene identified as encoding a carotenoid cleavage dioxygenase (CCD) was the maize *VIVIPAROUS14* (*Vp14*) gene which is required for the formation of abscisic acid (ABA), an important hormone that mediates responses to drought stress and aspects of plant development such as seed and bud dormancy (Zeevaart and Creelman 1988). The VP14 enzyme cleaves at the 11,12 position of the epoxycarotenoids 9′-*cis*-neoxanthin and/or 9-*cis*-violaxanthin and is now classified as a 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Schwartz *et al*. 1997), a subclass of the larger CCD family. Since the discovery of Vp14, many other CCDs have been shown to be involved in the production of a variety of apocarotenoids (Auldridge *et al*. 2006a). In insects, the visual pigment retinal is formed by oxidative cleavage of β-carotene by β-carotene-15,15′-dioxygenase (von Lintig and Vogt 2000). Retinal is produced by an orthologous enzyme in vertebrates, where it is also converted to retinoic acid, a regulator of differentiation during embryogenesis (Wyss *et al*. 2000). A distinct mammalian CCD is believed to cleave carotenoids asymmetrically at the 9,10 position (Kiefer *et al*. 2001) and, although its function is unclear, recent evidence suggests a role in the metabolism of dietary lycopene (Hu *et al*. 2007). By contrast, in flowering plants no 15,15′ cleave activity has been detected. In addition to NCED, the CCDs from plants are distributed into four classes: CCD1, CCD4, CCD7 and CCD8. CCD1 and CCD4 seem to be involved in the production of aroma volatiles. The first member of the CCD1 subfamily was identified from *Arabidopsis thaliana* (Schwartz *et al*. 2001). Sequence homology then allowed the identification and characterization of orthologs from several plant species, such as *Crocus sativus* (Bouvier *et al*. 2003; Rubio *et al*. 2008), *Lycopersicum sculentum* (Simkin *et al*. 2004a), *Vitis vinifera* (Mathieu *et al*. 2005), *Cucumis melo* (Ibdah *et al*. 2006), petunia (Simkin *et al*. 2004b), *Zea maize* (Vogel *et al*. 2008), *Fragaria* × *ananassa* (García-Limones *et al*. 2008), *Medicago truncatula* (Floss *et al*. 2009), *Oryza sativa* (Ilg *et al*. 2009) and *Rosa damascena* (Fong-Ching *et al*. 2009). CCD1 is a non-heme enzyme which uses oxygen to cleave a variety of carotenoid substrates symmetrically at the 9,10 and 9′,10′ positions of cyclic and acyclic carotenes. These generate C_{14} dialdehydes, which are common to all carotenoid substrates, and two variable end-group-derived C_{13} ketones. The wide substrate specificity of plant CCD1s allows the production of divergent volatile C_{13} compounds, including β-ionophores, α-ionones, pseudoionone and geranylacetone (Schwartz *et al*. 2001). The CCD1 enzymes also recognize the 5,6 or 5′,6′ bond positions of linear carotenes leading to the volatile C_8 ketone 6-methyl-5-hepten-2-one (Vogel *et al*. 2008), and the 7,8 and 7′,8′ double bonds of acyclic carotenoid ends leading to geranial (Ilg *et al*. 2009).

We have shown that CCD4 enzymes cleave double bonds at 9,10(9′,10′) positions, and seem to be more active than the CCD1 enzymes, at least for the β-carotene substrate (Rubio *et al*. 2008). Recently, the activity of other CCD4 enzymes have been characterized from apple (*Malus x domestica*, MdCCD4), chrysanthemum (*Chrysanthemum x morifolium*, CmCCD4a), rose (*Rosa x damascena*, RdCCD4), osmanthus (*Osmanthus fragrans*, OfCCD4), and *Arabidopsis*, AtCCD4 (Huang *et al*. 2009). CmCCD4a and MdCCD4 cleaved β-carotene to yield β-ionone, while

RdCCD4, and AtCCD4 cleaved 8'-apo-β-caroten-8'-al to yield β-ionone, which demonstrates that all the CCD4 enzymes cleave their substrates at 9,10 and 9',10' positions. Although CCD1 and CCD4 enzymes cleave carotenoids at the same positions (9,10 and 9',10′), CCD4 enzymes seem to be more substrate specific than CCD1. CCD4s could not cleave linear carotenoids such as lycopene and ζ-carotene, or carotenoids containing a hydroxyl group such as zeaxanthin and lutein. It seems that CCD4s only cleave cyclic non-polar carotenoids such as β-carotene. In addition, CCD4s are targeted at the plastids (Ytterberg *et al*. 2006; Rubio *et al*. 2008), whereas CCD1 enzymes are cytosolic and lack a chloroplast transient peptide in their sequences (Bouvier *et al*. 2003; Tan *et al*. 2003; Simkin *et al*. 2004a; Auldridge *et al*. 2006b). The plastid, or more exactly, the plastoglobule location of the CCD4 enzymes, allows these enzymes to obtain access to plastid carotenoids, while the CCD1 activity is limited to carotenoids outside these organelles or once these organelles have lost homeostasis or are targeted for degradation.

The CCD7 and CCD8 enzymes are implicated in the generation of the apocarotenoid hormone strigolactone involved in shoot branching. CCD7 and CCD8 are conserved across angiosperm species including monocotyledons: MAX3 (more axillary shoots), RMS5 (ramosus) and HTD1 (high tillering dwarf)/D17 encode (CCD7) (Sorefan *et al*. 2003; Booker *et al*. 2004, 2005; Johnson *et al*. 2006; Zou *et al*. 2006). Recombinant AtCCD7 exhibits regioselectivity for the 9,10 position similar to CCD1, yet it cleaves only once asymmetrically, resulting in C_{13} and C_{27} products (Schwartz *et al*. 2004). MAX4, RMS1, D10 and DAD1 (decreased apical dominance) encode another sub-class of CCDs designated as CCD8 (Snowden *et al*. 2005; Sorefan *et al*. 2006; Arite *et al*. 2007; Simons *et al*. 2007), and cleave the product generated by CCD7 (Schwartz *et al*. 2004; Alder *et al*. 2008), but are also able to act directly on carotenoid substrates (Auldridge *et al*. 2006b). Therefore, CCD7 and CCD8 might catalyse sequential carotenoid cleavage reactions, although further studies should be carried out to ascertain their role in plants.

REGULATION OF CAROTENOID BIOSYNTHESIS DURING STIGMA DEVELOPMENT IN SAFFRON

Since carotenoids are just one class of isoprenoids, the regulation of their formation must involve the co-ordinated flux of isoprenoid units (IPP) into the C_{40} carotenoids as well as the other branches of the isoprenoid pathway. In higher plants, the five-carbon building blocks of all terpenoids, IPP and dimethylallyl diphosphate, are derived from two independent pathways localized in different cellular compartments: the MEP or nonmevalonate pathway, localized in the plastids, and the cytosol-localized mevalonate pathway (MVA) (**Fig. 2**). The MEP pathway furnishes the formation of monoterpene-, diterpene- and carotenoids (Lichtenthaler *et al*. 1997; Rhomer 1999). In snapdragon flowers it provides both monoterpene and sesquiterpene formation (Dudareva *et al*. 2005). During the development of *C. sativus* stigmas, the 1-deoxylulose-5-phosphate synthase, DXS, which is the first enzyme specific to the MEP pathway, is highly expressed in all developmental stages, whereas the 3-hydroxy-3-methylglutaryl CoA reductase, HMGR, the enzyme which catalysed the third step of the MVA pathway, is expressed at low levels (Rubio *et al*. 2009), suggesting that DXS plays an important role in the control of isoprenoid biosynthesis in the stigma tissue, characterized by high levels of carotenoid derivatives.

Carotenogenesis in ripening fruit and petals has been studied extensively (Hugueney *et al*. 1996; Hirschberg 2001; Moehs *et al*. 2001; Zhu *et al*. 2002, 2003; Kato *et al*. 2004). In these tissues development and carotenoid accumulation parallels chloroplast to chromoplast transition. In *C. sativus*, however, the development of the stigma occurs concomitantly with the amyloplast to chromoplast transition and the stigma never turns green during this process. Chromoplasts in *C. sativus* have a tubular structure and show numerous plastoglobules and vesicles (Grilli-Caiola and Canini 2004). Stigma development also parallels carotenoid accumulation (Castillo *et al*. 2005), making this tissue a good model system to study carotenoid formation and accumulation during this transition process. Carotenoid accumulation that occurs in the transition of green to red in tomato fruit chromoplasts is mediated by transcriptional regulation of *PSY* and *PDS*. In *C. sativus* the transcript levels of these carotenogenic genes, *CsPSY* and *CsPDS,* are relatively low and modulated during development, displaying their lowest levels at early developmental stages and the highest in the red and preanthesis stages. In contrast, the transcript levels of the chromoplast-specific lycopene cyclase, *CstLcyB2a*, and the chromoplast specific carotene hydroxylase, together with the *CsCHY1* genes, are much higher and accumulate in the red and scarlet stages of saffron stigmas, which is consistent with the production and accumulation in this tissue of β-carotene and zeaxanthin, the main carotenoids present in stigma extract and the precursors of saffron apocarotenoids (Castillo *et al*. 2005; Ahrazem *et al*. 2009). Moreover, the analysis of the stigmas of several *Crocus* species showed that quantitative and qualitative changes in the carotenoid pigments were related to the expression levels of *CstLcyB2a* and *CsCHY1*, thus supporting the hypothesis that the major mechanism controlling carotenoid formation in *C. sativus* is transcriptionally regulated at the level of both genes. All these data suggest that the reactions catalysed by CstLcyB2a and CHY1Cs enzymes could be the limiting steps in the formation of saffron apocarotenoids in the stigma tissue, due to the accumulation of the respective substrates and products. However, the levels of carotenoids in the developed stigmas are much lower compared with the massive accumulation of apocarotenoids, suggesting a high flux rate in the carotenoid pathway and an important role for the carotenoid cleavage dioxygenases.

REGULATION OF APOCAROTENOID BIOSYNTHESIS DURING STIGMA DEVELOPMENT IN SAFFRON

The flavour of a particular food or ingredient can be thought of as the sum of a complex interaction between taste receptors, the ortho- and retronasal olfactory systems, mouth texture, and visual appearance (Shepherd 2006). Saffron constitutes a complex mixture of volatile and non-volatile compounds that contribute to the overall aroma and flavour of this condiment (Tarantilis and Polissiou 1997). The major components of saffron are the apocarotenoids *cis*- and *trans*-crocins, picrocrocin (β-D-glucopyranoside of

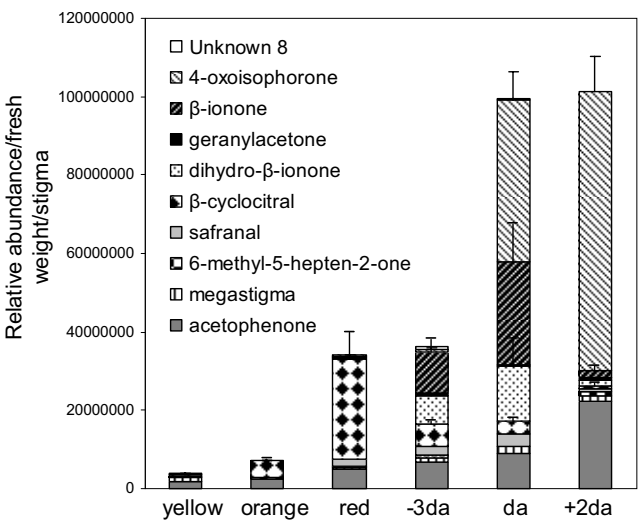

Fig. 4 Relative levels of apocarotenoid volatiles emitted during different stages of stigma development. Three days before anthesis (-3da), day of anthesis (da), and two days after anthesis (+2da).

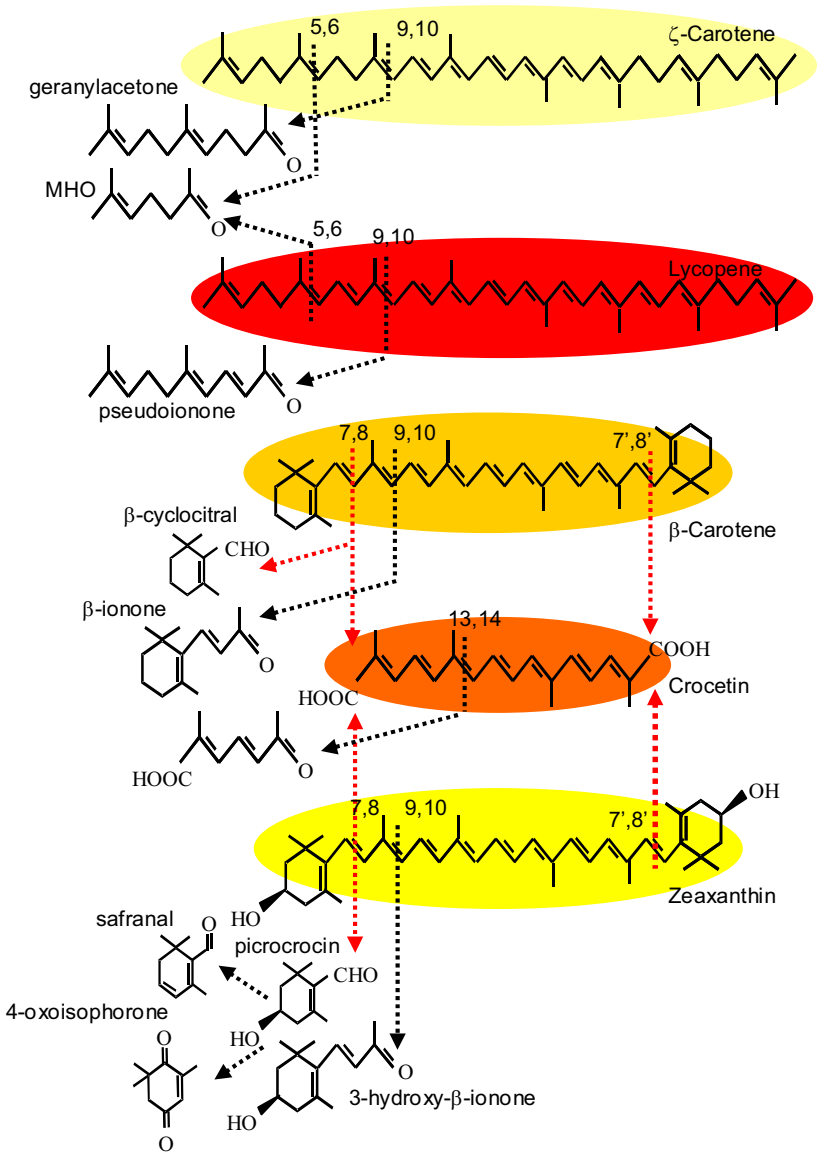

Fig. 5 Scheme for the reactions potentially catalyzed by CCD proteins in *C. sativus* stigmas. The carotenoid substrates (right) when cleaved would yield two monoaldehydes and a central dialdehyde product (left). MHO, 6-methyl-5-hepten-2-one.

hydroxyl-β-cyclocitral), and its degradation product, the odour-active safranal (Kanakis *et al*. 1994). However, recent studies reveal a different volatile composition in unprocessed tissue (Rubio *et al*. 2008, 2009), suggesting the presence of a degradation process responsible for the organoleptic properties of saffron from preformed apocarotenoid compounds (Dauria *et al*. 2006), as has also been observed in other spices (Mookherjee *et al*. 1990). In addition to crocetin, picrocrocin, and its degradation product, safranal, other minor apocarotenoid volatiles have been found in the stigma tissue (Rubio *et al*. 2008, 2009). Although in minor amounts, these are sufficient to have an impact on human perception (Goff and Klee 2006). The levels of these volatiles increases as stigma develops (**Fig. 4**), suggesting an involvement with pollinator attraction (Rubio *et al*. 2008). Based on their chemical structures these compounds are likely products of oxidative carotenoid cleavage. The potential relationships between these volatiles and their carotenoid precursors following 5,6 or 5',6', 7,8 or 7',8', and 9,10 or 9',10' oxidative cleavage are shown in **Fig. 5**.

As previously mentioned, the CCD1 enzymes have broad substrate specificity, cleaving multiple linear and cyclic carotenoids at either the 5,6 or 9,10 double bond positions, and CCD4 enzymes are active over β-carotene recognizing 9,10 and 9',10' positions. Two CCD1 enzymes have been isolated from *C. sativus* (Bouvier *et al*. 2003; Rubio *et al*. 2008). *CsCCD1a* expression, however, remains practically constant throughout stigma development, with no expression in the senescent stigmas. The *CsCCD1b* gene reached maximum expression in the earlier stages and its expression dropped in the scarlet stages. By contrast, *CsCCD4a* and *b* reached the maximum expression levels in the scarlet stages (Rubio *et al*. 2009) which coincide with the highest levels of their reaction products.

The biogenesis of the main colour principles, crocins, and safranal, was proposed to be derived by bio-oxidative cleavage of zeaxanthin (Pfander and Schurtenberger 1982) by a 7,8-7′,8′ cleavage reaction. This reaction was found to be catalysed by a 7,8-7′,8′ CCD (CsZCD, zeaxanthin cleavage dioxigenase) obtained by cloning the *CsZCD* gene from *C. sativus*. CsZCD specifically catalyses the formation of crocetin dialdehyde and two molecules of 3-hydroxy-β-cyclocitral from zeaxanthin (Bouvier *et al*. 2003). Recently, two genes coding for CCD enzymes have been isolated and characterized from *C. sativus*, *CsCCD4a* and *CsCCD4b*. CsCCD4a (580 aa) and CsCCD4b (569 aa) showed 98% similarity in 369 amino acids with the previously isolated CsZCD (369 aa) (Bouvier *et al*. 2003). However, CsCCD4a and b are more than 200 amino acids longer than CsZCD, indicating that most likely the previously reported sequence represents an N-t truncated version. Furthermore, CsZCD lacks important residues and domains for the dioxygenase activity (Kloer *et al*. 2005; Kloer and Schulz 2006). CsCCD4a and CsCCD4b have a 9,10(9′,10′) cleavage acti-

vity and since they did not display the expected 7,8(7′,8′) activity for crocetin formation, these enzymes are most probably involved in the generation of β-ionone during stigma development (Rubio *et al*. 2008). Recent studies show the biogenesis of crocetin from β-carotene and zeaxanthin (Rubio *et al*. 2009). The volatile β-cyclocitral is generated by the cleavage of β-carotene at the 7,8 (7′,8′) positions, and is detected during stigma development of *C. sativus*. Therefore, the dioxygenase cleavage enzyme involved in crocetin biosynthesis should be able to recognize β-carotene and zeaxanthin as substrates.

GLUCOSYLATION OF SAFFRON APOCAROTENOIDS

The final step in the biosynthesis of the 20-carbon esterified carotenoid crocin is the transformation of the insoluble crocetin into a soluble and stable storage by glucosylation. The enzymes leading to glycoside formation, the glycosyltransferases (GTs), transfer nucleotide-diphosphate-activated sugars to low molecular weight substrates. A broad range of different carbohydrate moieties can be added, recruiting all forms of sugars independently (monoglycosides), in parallel, or in chains (di-, tri-glycosides, etc.). This gives rise to a broad spectrum of glycosidic structures for any given aglycone. The GTs are encoded by large multigene families and can be identified by a signature motif in their primary sequence (Hughes and Hughes 1994), which classifies them as a subset of Family 1 GTs. There is a need for isolating and characterizing the specific enzyme that transfers or hydrolyzes a β-glucoside whose aglycone moiety is of interest in food quality. Such biochemical data are crucial when making practical decisions as to whether or not enzymes from host plants or other sources should be added to drinks and beverages before, during or after processing in order to enhance flavour, aroma and other quality factors. Likewise, such data are essential for targeting enzymes with desirable properties for overproduction in transgenic microbial or plant hosts and improvement of their catalytic properties and stability for specific uses by genetic engineering.

A pathway for glucosylation of encapsulated crocetin was proposed in 1997 by Dufresne *et al*., who indicated that crocetin glucosylation into crocin is sequential and may involve two different glucosyltransferase activities. A UDP-glucose: crocetinglucosylester 6"-*O*-glucosyltransferase involved in the glucosylation of the carboxylic end of crocetin and an uridine-5'-diphosphoglucose (UDP-glucose)-crocetin 8,8'-glucosyltransferase catalysing the glucosylation of hydroxyl groups of the glucose already linked to the aglycone with formation of gentiobiosyl esters (Côte *et al*. 2000, 2001). A molecular biology approach allowed the isolation of *UGTCs2*, a glucosyltransferase enzyme involved in crocin and crocetin glucosylation in *C. sativus* stigmas (Rubio *et al*. 2004). Concomitant with stigma development, the accumulation of crocins of higher glucose content takes place (Rubio *et al*. 2009), a result which agrees with the expression patterns observed for *UGTCs2*. The *in vitro* results with the recombinant enzyme UGTCs2 showed that UGTCs2 activity promotes the formation of highly glucosylated crocetin esters using crocetin, crocetin β-D-glucosyl ester and crocetin β-D-gentibiosyl ester, thus indicating that the enzyme displays *in vitro* the two types of activities described by Dufresne *et al*. (1997). Furthermore, the enzyme was able to form a pigment more polar than crocin that was not detected in saffron stigmas. Similarly, Dufresne *et al*. (1999), using *C. sativus* cell suspensions supplemented with crocetin, detected the presence of more polar crocetin glucosides as major products that were not detected in stigma tissue.

Besides crocins and picrocrocin, other glucosylated apocarotenoids have been identified in *C. sativus*, but at low levels. The list includes a monogentiobiosyl ester, structurally related with crocin, which could be generated by the oxidative cleavage of zeaxanthin at the positions 7,8 and 13,14 (Straubinger *et al*. 1997), the β-D-glucopyranosides (4R)-4-hidroxy-3,5,5-termetilciclohex-2-enone, (4S)-4-hydroxy-3,5,5-termetilciclohex-2-enone, and the (4S)-4-hydroxymethyl-3,5,5-termethylcyclohex-2-enone, along with seven more apocarotenoids related to other compounds previously identified in various plant species (Straubinger *et al*. 1998). Thus, different glucosyltransferase activities should be responsible for the glucosylation of these compounds. In fact, several EST clones from the Saffron Gene Database (D'Agostino *et al*. 2007) encode putative glucosyltransferase enzymes, which will be of interest in determining the function and substrate specificity of these enzymes.

OTHER PLAYERS INVOLVED IN CAROTENOID ACCUMULATION

Despite significant progress concerning carotenogenesis in plants, the control mechanisms regulating overall carotenoid biosynthesis and accumulation remain an enigma. Although news strategies such as combinatorial genetic transformation, allowed to gain insight into the bottlenecks in the carotenoid pathway (Zhu *et al*. 2008). However, the regulatory genes that positively or negatively modulate carotenogenesis and the regulatory factors that govern carotenogenic gene expression in plants remain unknown. Mutant analysis allowed the isolation of the *Or* gene (Lu *et al*. 2006), which encodes a plastid-associated protein containing a Cys-rich domain found in DnaJ-like molecular chaperones and is expressed highly in tissues normally rich in proplastids or noncoloured plastids. Rather than directly regulating carotenoid biosynthesis, the *Or* gene controls carotenoid accumulation by inducing the formation of chromoplasts, which provide a metabolic sink to sequester and deposit carotenoids (Li and Van Eck 2007). Highly conserved *Or*-like open reading frames are found in several higher plant lineages, including *C. sativus* (D'Agostino *et al*. 2007) where they could be involved in apocarotenoid accumulation in the stigma tissue.

CONCLUSIONS

Since the elucidation of the carotenogenic pathway in plants, there has been a steady increase in understanding the complexities which regulate this pathway. Much of this work has been done in plants characterized by the massive accumulation of carotenoids in their fruits, and much less is known about other interesting plants such as saffron, where apocarotenoid metabolism is responsible for the economic value of this plant. Thus, the isolation and biochemical analysis of genes involved in the carotenoid metabolism should be of major interest, in addition to the measurements of flux coefficients, metabolite channelling and the interactions between carotenogenic enzymes and other protein partners. Far more information is needed before the control mechanisms of apocarotenoid accumulation in stigma tissue can be fully understood.

ACKNOWLEDGEMENT

We are grateful for support from the BIO2003-05259, BIO2006-00841 and PAI08-0211-6481 projects.

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How to reference: Author name(s) (2010) Title of chapter. In: Husaini AM (Ed) Saffron. Global Science Books, UK, pp. X-XX

Potential Healthy Effects of Saffron Spice (*Crocus sativus* L. Stigmas) Consumption

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ABSTRACT

Saffron (*Crocus sativus* L.), has been used since ancient ages in food for its flavouring, aromatic and colouring properties but also for its biomedical activity. In the past years many efforts have been made in order to demonstrate scientifically the healthy effects attributed to saffron consumption since Dioscorides' time. More than 400 papers have been published in the last decade related to antioxidant properties, cancer, neuronal injury and sedative effect, among others. It has been found that its antioxidant activity is the major responsible for many of the properties that helps to prevent or diminish some diseases. But the majority of these research use animals, making difficult to understand the human application. In this review, a first attempt to translate animal doses to human intake when saffron is included on the diet is carried out, in order to make an estimation of the potential healthy effects in humans.

Keywords: antioxidant properties, *Crocus sativus* L., healthy effects, human equivalent doses, saffron intake
Abbreviations: b.w., body weight; **BSA**, body surface area; **HED**, human equivalent dose; **MI**, myocardial infarction; **PMS**, premenstrual syndrome

CONTENTS

INTRODUCTION

Since ancient ages, spices have placed a major role in cooking, cosmetics, perfumery, global exploration, economics and medicine (Dog 2006). Saffron (*Crocus sativus* L.), is an example of a multi-purpose spice widely used for many centuries. Starting in Mesopotamia, where saffron was used in religious celebrations and for curative purposes; continuing with Phoenicians, where used it to dye cloths, and in ancient Rome, used as a treatment and dye, as well as in perfumes and ointments (Giaccio 2004; Carmona *et al*. 2006). Also used by Cleopatra (69-30 B.C.), it was a cosmetic, phitotherapy and a nail, hair and lips dye. Healing properties of saffron are well known since ancient times, as said by Dioscorides Pedacio, a Greek medical practitioner of the first century, who considered it as sexual stimulant, anti-inflammatory and as a drunkenness impediment. Since then, saffron has been considered as anodyne, antidepressant, a respiratory decongestant, antispasmodic, aphrodisiac, diaphoretic, emmenagogue, expectorant and sedative, among others (Abdullaev and Espinosa-Aguirre 2004).

Recently, research on saffron properties has covered a great interest, demonstrated by the increase of the number of publications in scopus and science direct databases, as shown in **Fig. 1**, where it can be observed the exponential augment, especially from 1996. Approximately, every 2 years, publications duplicate its number, being in 2009 about 5 times more than in 2000. Many reviews have been published in the past recent years (Deng *et al*. 2002; Abdullaev and Espinosa-Aguirre 2004; Schmidt *et al*. 2007; Soeda *et al*. 2007; Kianbakht 2008), but some properties of saffron have been particularly investigated, as seen in **Fig. 2**, where antioxidant, nervous system damage and cancer properties cover a great number of publications, almost 3 to 5 times more than the rest, follow by cardiovascular injury and antinociceptive effects.

The current paper provides an overview of saffron investigations on its biological activity and diseases preven-

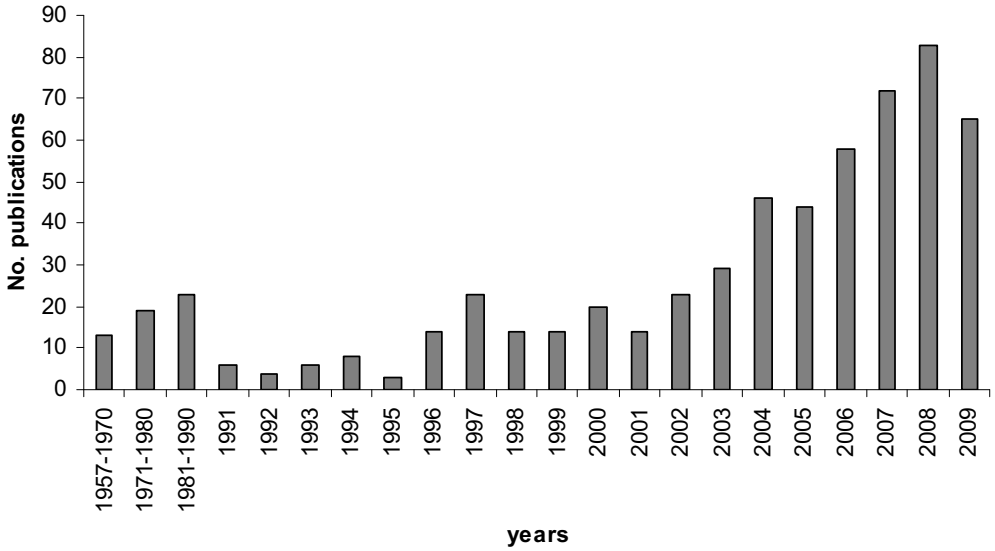


Fig. 1 Biomedical properties of saffron publications during the past years (based on scopus.com and sciencedirect.com).

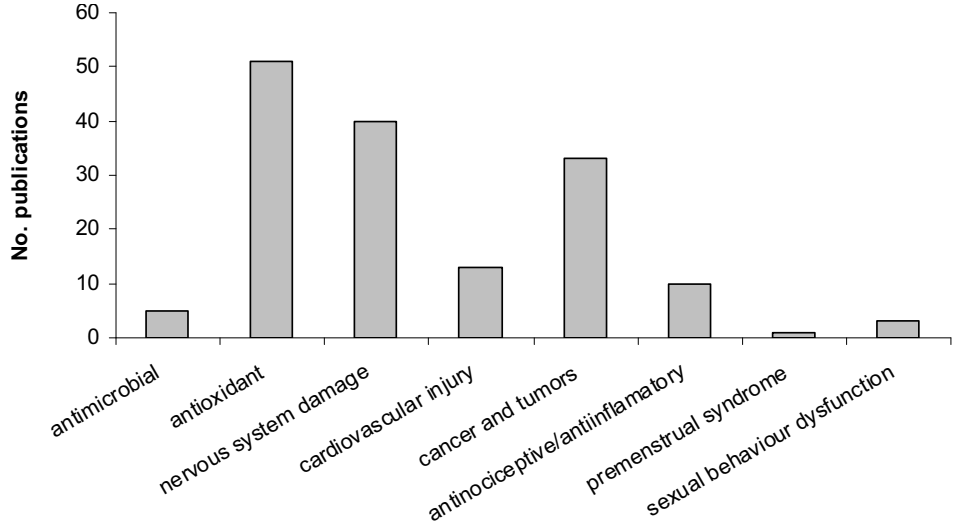


Fig. 2 Principal saffron properties investigated during the past decade (based on scopus.com and sciencedirect.com).

tion during the past decade. In addition, an attempt to relate saffron consumption with its potential healthy benefits when added to different food, so that, the effects found to be effective on animals, will be estimated in humans.

BIOLOGICAL ACTIVITIES OF SAFFRON

The main biological activity of saffron is based on its great antioxidant ability; in fact the antioxidant properties of saffron are well known and have been widely studied since this property is responsible for many of its biomedical attributes. A radical scavenging activity is involved in aging processes, anti-inflammatory, anticancer and wound healing activities, among others (Assimopoulou *et al.* 2005), so many efforts have been made in order to find natural products that posses this property. Assimopoulou *et al.* (2005) suggests that saffron could be used in functional foods, drinks with antioxidant activity and in pharmaceutical and cosmetic preparations, as well as, food supplement with antioxidant properties. Saffron extracts exhibited a remarkable intracellular antioxidant activity. Moreover, the antioxidant efficiency observed in ethanol saffron extracts was equivalent to 116 mg α-tocopherol/g (Chen *et al*. 2008). So that, it can be assumed that this property is responsible for preventing many diseases which mechanisms involve oxidation, such as neurodegenerative injury (Urrutia *et al*. 2007) and cardiovascular diseases, which are described below and injury in kidney or brain tissues caused by ischemia-reperfusion (I/R) (Hosseinzadeh *et al*. 2007b). In addition, treating thermal induced burn wounds with saffron extract cream (20%) result in a significantly increased re-epithelialization that could be explained for the antioxidant effects of this spice (Khorasani *et al.* 2008).

Other important property which converts saffron in a beneficial spice for health is their antimicrobial activity. This one has been studied under different saffron parts; it is well known that many spices such as garlic and basil are antibacterial agents (Low Dog 2006). Ethyl acetate extracts of stigma, stamen and leaves were tested on *Sthaphylococcus aureus*, *Staphylococcus epidermis*, *Escherichia coli*, *Micrococcus luteus*, *Candida albicans*, *Cladosporium* sp. and *Aspergillus niger*, finding that the leave extract did not show antimicrobial activity at a concentration of 100 mg/ml. The antifungal activity of stigma was higher than stamen; in contrast, the antibacterial activity of stamen was higher than the rest of the parts studied (Vahidi *et al*. 2002).

On the other hand, the anti-*Helicobacter pylori* activity of saffron extracts, safranal and crocin was investigated using aqueous and methanol extracts and four antibiotics as control. All isolates were susceptible to methanol and aqueous saffron extracts, being the minimum inhibitory concentrations of methanol saffron extract, crocin and safranal 677, 26.5 and 16.6 µg/ml, respectively (Nakhaei *et al*. 2008). In other series of studies were determined other antiulcer properties of saffron, suggesting that saffron inhibits gastric acid secretion and stimulates mucus secretion which is a

barrier to prevent damage (Al-Mofleh *et al*. 2006). In this work saffron extracts at 250 mg/kg b.w. produce a significant decrease in the volume of gastric secretion and ulcer index in the animals tested. In addition, Kianbakht and Mozaffari (2009) studied the effects of pretreated rats with saffron extract (25, 100 and 250 mg/kg b.w.), crocin (2.5, 5 and 10 mg/kg b.w.) and safranal (0.25, 2 and 5 ml/kg b.w.), finding that these extracts, prevent gastric lesions, increase lipid peroxidation and decrease glutathione levels induced by indomethacin, effects that are comparable to omeprazole, an inhibitor protons pump, which is used as an antiulcerogenic agent.

These properties of saffron could be applied as possible therapeutic agent for a several diseases as demonstrated by several biomedical studies.

BIOMEDICAL STUDIES WITH SAFFRON

Nervous system damage

1. Neuronal injury

Saffron and its constituents: crocetin glycosides and picrocrocin were demonstrated to cause protective effects on neuronal injury acting as an antioxidant. Crocin, among the rest of the components, results the most potent antioxidant, capable of combating ischemic stress-induced neuron death (Saleem *et al*. 2006; Ochiai *et al*. 2007). In addition, 727.5 mg/kg b.w. of safranal in rats showed protective effects on hippocampal tissue from rats under ischemic conditions, elevating antioxidant capacity of the hippocampus (Hosseinzadeh and Sadeghnia 2005). Ochia *et al.* (2004) suggest that crocin inhibits apoptosis in a model cellular for neuronal differentiation, PC-12 cell line, and combats the serum/glucose deprivation-induced ceramide formation in PC-12 cells by increasing glutathione (GSH) levels and preventing the activation of a pathway for neural cell death.

2. Diabetic neuropathy

Diabetic neuropathy is one of the most frequent complications of diabetes. Vascular and neural diseases are closely related; in fact microvascular dysfunction occurs together with the progression of neural dysfunction. Neuronal ischemia is a well-established characteristic of diabetic neuropathy. The mechanisms of neurotoxicity from high glucose levels are poorly understood, but an increase on reactive oxygen species has been proposed as possible mechanism. Saffron, as antioxidant, can have neuroprotective effects. Saffron extracts and crocin were studied in glucose-induced neurotoxicity, using PC12 cells as a suitable *in vitro* model of diabetic neuropathy, showing that saffron extract (5 and 25 mg/ml) and crocin (10 and 50 μM) could decrease the toxicity caused by glucose, suggesting that saffron and crocin could be potentially useful in diabetic neuropathy treatment (Mousavi *et al.* 2009).

3. Retinal function

Recently, it was published by Maccarone *et al*. (2008) that saffron and carotene extracts (1 mg/kg b.w. /d) as feed supplementation in rats, mitigates retinal damage induced by exposure to continuous bright light (1000 lux) during 24 hrs. They mentioned that the antiapoptotic characteristic of saffron makes it interesting in the treatment of retinal neurodegenerative disease; moreover, it reduces photoreceptor death induced by environmental stresses. In another study using retinal cell cultures from bovine and primate eyes, crocin protected the photoreceptors against blue light or white light-mediated damage in a concentration dependent manner (10–160 μM) (Laabich *et al.* 2006). Finally, saffron can significantly inhibit the elevation of glutamic acid concentration, fact that contributes to neurodegeneration of retina, thus, prevents retina damage (Yang X-G *et al.* 2006). For a different type of retinal malfunction, such as ischemic retinopathy and age-related macular degeneration, which are the leading ocular diseases that cause blindness, it has been studied that crocin analogs increase the blood flow in the retina and choroid and facilitate retinal function recovery, leading to the conclusion that crocin analogs could be used to treat this problem (Xuan *et al.* 1999).

4. Alzheimer's disease

Alzheimer's disease is the most common form of dementia among people over 65 years old which is characterized by cognitive impairment and memory deterioration, promoted by deposition of amyloid *β*-peptide (A*β*) fibrils that is caused by oxidation. Thus, to identify agents inhibiting the pathogenesis of Alzheimer's disease, the antioxidant properties of *C. sativus* were examined on A*β* fibrils and compared with that of tomato and carrot by Papandreou *et al*. (2006). The results showed that saffron extracts at concentrations of 300 and 600 μg/ml had twice the antioxidant activity than tomato and carrot extracts. In addition, *C. sativus* stigmas extract significantly inhibited the formation of amyloid fibrils in a concentration and time-dependent manner. In conclusion, the study resulted to demonstrate that saffron extract has antioxidant and antiamyloidogenic activity; as a result, it has a positive effect on cognitive function, indicating that saffron may be valuable for prevention or delay of Alzheimer's disease. Recently, a clinical trial with 54 patients of 55 years old or older, with mild-to-moderate Alzheimer's disease, using a saffron capsule of 30 mg/day, provides preliminary evidence of saffron possible therapeutic effect (Akhondzadeh *et al.* 2010).

5. Parkinson's disease

Parkinson's disease is a terminal, progressive neurodegenerative disorder. A cure has not been developed yet, so many efforts for relief the symptoms have been done. The causes of the disease are marked by generation of excessive free radicals but the exact mechanism is still unclear (Ahmad AS *et al.* 2005; Ahmad M *et al.* 2005; von Bohlen und Halbach *et al.* 2005). The neuromodulatory effects of crocetin (75 μg/kg b.w.) were studied resulting in a neuronal protection from a catecholaminergic neurotoxin that causes loss of cells in the substantia nigra (Ahmad AS *et al.* 2005), mechanism that could be helpful for reducing Parkinson.

6. Seizures

Since traditional medicine, saffron has been used as anticonvulsant agent, but its mechanisms of action deserve further study. Seizures are produced when neurons are activated in an unusually synchronous manner, disturbing the balance between excitation and inhibition and altering several classic neurotransmitters systems such as the glycine, glutamatergic and GABAergic (Engelborghs *et al.* 2000). Depressant effects on the central nervous system are at least partly responsible for inhibiting the alterations mentioned above. Given that the current therapeutic treatment using antiepileptic drugs is associated with side-effects, plants, such as saffron, would be helpful in this treatment, as shown by Hosseinzadeh and Khosravan (2002), who found that ethanolic (0.2-2.0 g/kg b.w.) and aqueous (0.08-0.80 g/kg b.w.) saffron extracts increased the latency of convulsions induced by pentylenetetrazol (PTZ), a popular chemoconvulsant, in a dose-dependent manner and decreased the duration of tonic seizures caused by electroshock. Safranal (0.15-0.35 mg/kg b.w.) showed anticonvulsant behaviour as well, in PTZ-induced seizures (Hosseinzadeh and Talebzadeh 2005). Besides, Hosseinzadeh and Sadeghnia (2007) studied deeply these properties of safranal showing that peripheral administration of safranal (72.75, 145.5 and 291 mg/kg b.w.) exerts a dose dependent decrease in minimal clonic seizure (MCS) induced by PTZ and first generalized tonic-clonic seizures (GTCS). The exact mechanisms of saffron action are unclear yet.

7. Learning behaviour

Several studies have reported that saffron extracts and two of its main ingredients crocin and crocetin, improved memory and learning skills in ethanol-induced learning behavior impairments in mice and rats (Sugiura *et al.* 1994; Abe *et al.* 1999; Abe and Saito 2000), suggesting that oral administration of saffron may be useful as treatment for neurodegenerative disorders and related memory impairment. Recently, rats treated with 30 and 60 mg/kg b.w. of saffron extracts were capable of discriminate between familiar and novel objects (Pitsikas and Sakellaridis 2006), finding the enhancing effects of crocetin esters on memory and its implication in the mechanisms underlying recognition and spatial memory (Pitsikas *et al.* 2007).

8. Anxiety

The traditional therapeutic potential of crocetin esters in anxiety was investigated using a light/dark chamber test in rats. The results showed that crocin at 50 mg/kg b.w. reduced the anxiety of animals but the mechanism that might account for this effect was not determined (Pitsikas *et al.* 2008). In addition, the anxiolytic and hypnotic effects of saffron (56, 80, 320 and 560 mg/kg b.w.) and safranal (0.15 and 0.35 ml/kg b.w.) were similar to diazepam, which is used in pharmacology as a sedative. Safranal was confirmed as anxiolytic in a dose-dependent manner (Hosseinzadeh and Noraei 2009).

9. Sedative/relaxant

The sedative effects of saffron are well known since traditional medicine, and it was confirm by Boskabady and Aslani (2006). Aqueous-ethanolic saffron extract (0.15-0.6%g) and safranal (0.15-0.60 ml containing 0.2 mg/ml solution) showed a potent relaxant effect that is comparable or even higher than theophylline, a relaxing drug. To corroborate the mechanism of action, another study was published, suggesting that relaxation is due to saffron stimulatory effects on β-adrenergic receptors being superior to its agonist's available (Nemati *et al.* 2008). β-adrenoceptors agonists, such as saffron, stimulate the liver, kidneys, increase heart rate and heart contractility rate (Boskabady *et al.* 2008), vasodilatation due to petals (Fatehi *et al.* 2003) and bronchodilation, to which can be attributed the proven antitussive effect of safranal and ethanolic extracts of saffron stigma (Hosseinzadeh and Ghenaati 2006). Relaxant properties of saffron could be useful for treating different conditions described below.

10. Depression

Herbal treatments, including saffron, as antidepressant agents have been widely studied. There is strong evidence that, stigmas, petals, safranal and crocetin esters of saffron exert an antidepressant activity. Since a few years ago, efforts has been made by some research groups, especially in Iran, in order to know the doses of the different extracts that can be useful to treat this disorder. It was observed during 6 weeks 30 patients, that if saffron (30 mg/day) is compared with imipramine (100 mg/day), a antidepressant drug, saffron could be of therapeutic benefit in the treatment to mild to moderate depression (Akhondzadeh *et al.* 2004). As well as safranal (0.15-0.5 mg/kg b.w.) and crocin (50-600 mg/kg b.w.), that were proved to be effective on mice (Karimi *et al.* 2001). Fluoxetine activity, which is a common drug used for treating this disorder, can be compared with aqueous and ethanolic saffron extracts and with kaempferol obtained from saffron petals (Hosseinzadeh *et al.* 2004, 2007a). In the same way, the effect of kaempferol has been studied in 40 depressed patients (between 18 and 55 years) concluding that a treatment of 30 mg/day of a petal extract during 8 weeks and 30 mg/day of a stigma extract during 6 weeks can be helpful for treating this condition (Noorbala *et al.* 2004, 2005; Moshiri *et al.* 2006; Akhondzadeh *et al.* 2007). Finally, Akhondzadeh *et al.* (2008) concluded that, being petals less expensive than stigmas and exerting the same activity could represent a new alternative treatment.

Cardiovascular injury

1. Atherosclerosis

Hyperlipidemia is characterized for abnormal levels of lipids or lipoproteins in the blood stream causing thickness of the arteries' wall leading to a cardiovascular disease named atherosclerosis. Since several efforts have been made in order to know more about this mechanism and its prevention, the possibility of using antioxidants, such as crocin, as an inhibitor of this disease has been evaluated. There is evidence that crocin (25, 50 and 100 mg/kg b.w.) decrease greatly the content of cholesterol, triglyceride and density lipoprotein in blood and increase the content of high density lipoprotein (He *et al.* 2005; Xu *et al.* 2005). Moreover, thiobarbituric acid reactive substances decrease and plasma lipid levels remain unchanged in high lipid diet rabbits (Zheng *et al.* 2006). Sheng *et al.* (2006) confirmed that crocin (25, 50 and 100 mg/kg b.w.) significantly reduced serum triglyceride, total cholesterol, LDL cholesterol and VLDL cholesterol. In the same way, crocin suppressed the absorption of fat and cholesterol. In addition, crocetin can prevent the adhesion of leukocyte to bovine endothelial cells (BEC), which is important because adhesion and migration of leukocyte to endothelial cells is one of the early key steps in the atherosclerosis. This activity may be related to the antioxidant properties of saffron and protection for mitochondrion (Xiang *et al.* 2006). Furthermore, Sheng *et al.* (2006) found that the hypolipidemic effect of crocin was due to its inhibition of pancreatic lipase activity, being this enzyme the key to digestion and absorption of fat, so much effort has been directed to search an inhibitor. Crocin doses from 0.1 to 10,000 μg/ml, result in a dose-dependent, reversible inhibition of lipase that was more potent than the inhibition of gastric lipase (Sheng *et al.* 2006). Recently, another study revealed that saffron had superior hypolipidemic effect than crocin (Asdaq *et al.* 2009).

2. Myocardial infarction

Myocardial infarction (MI) is an acute condition of necrosis of the myocardium that occurs as a result of imbalance between myocardial demand and coronary blood supply. It is well established that reactive oxygen species have been implicated in the pathophysiology of MI and antioxidants suppress its formation. Therefore, the effects of crocin in cardiotoxicity isoproterenol induced were studied. Crocin at 20 mg/kg b.w./day, administered during 21 days, significantly modulated hemodynamic and antioxidant derangements, suggesting a cardioprotective effect through modulation of oxidative stress in such a way that maintains the redox status of the cell (Goyal *et al.* 2010; Joukar *et al.* 2010). In adittion, crocetin has beneficial effects on blocking inflammatory cascades caused by hemorrhage/resuscitation on cardiac injury at doses of 50 mg/kg b.w. (Yan *et al.* 2010).

3. Peripheral vascular diseases

It has been reported that the platelet-rich thrombi are the indispensable sources of thromboembolic complications, such as atherosclerosis, heart attacks, strokes, and peripheral vascular diseases. Therefore, inhibition of platelet functions represents a promising approach for the prevention and treatment of cardiovascular diseases, such as thrombosis. Crocetin effects on platelet activity and thrombosis formation were demonstrated showing a dose-dependent inhibition of platelet aggregation and significantly attenuation of dense granule release, as well as, prolonged the occlusive time in electrical stimulation-induced carotid arterial throm-

bosis. These findings suggest that the favourable impacts of crocetin on platelet activity and thrombosis formation may be related to the inhibition of Ca_2 elevation in stimulated platelets (Yang *et al.* 2008). In accordance with these results, other study using blood from healthy volunteers evaluated the inhibitory activity of saffron extract on human platelets, confirming a dose-dependent inhibition (Jessie and Krishnakantha 2005).

4. Insulin resistance

Insulin resistance is a condition in which normal levels of insulin are inadequate to produce a normal insulin response, situation that is linked to genetic and environmental factors, causing hyperinsulinemia, hypertension, dyslipidemia and being one of the principal factors for developing Diabetes mellitus type 2, which may lead in a cardiovascular disease. Crocetin at doses of 20 mg/kg b.w. and specially 40 mg/kg b.w. is capable of attenuate the development of insulin resistance and the abnormalities mentioned above, as well as, restoring free fatty acid metabolism disorders, which may explain the biochemical and nutritional basis of its inhibitory action (Xi *et al.* 2007).

Cancer and tumours

Chemoprevention is defined as the use of natural or synthetic agents to prevent of block the development of cancer. The chemopreventive and antitumoral potential properties of saffron and several other spices against cancer have been extensively studied during the last decade, proposing different hypotheses for the mode of action of its constituents. The cytotoxic effect of saffron extract (200-2000 µg/ml) was evaluated by Tavakkol-Afshari *et al*. (2008) in HepG2 and HeLa malignant cell lines, resulting in a decrease of viability of malignant cells in a concentration and time-dependent manner, fact confirmed by Feizzadeh *et al.* (2008). Saffron doses inducing 50% cell growth inhibition (IC50) values against HeLa and HepG2 were determined as 800 and 950 µg/ml after 48 hrs, respectively. It was concluded that saffron can cause cell death in which apoptosis or programmed cell death plays an important role (Tavakkol-Afshari *et al.* 2008). The cytotoxic and antitumor properties of saffron petals have been also studied, being the IC50 values of stigma and petal extract against tumour, 5.3 and 10.8 mg/ml (Hosseinzadeh *et al.* 2005), respectively. On the other hand, the genotoxic potential of anti-tumour drugs limits their efficacy in the treatment of cancers, so a study was designed to ascertain the chemoprotective potential of saffron against the genotoxicity of cisplatin, cyclophosphamide and mitomycin, three well known antitumor drugs. Saffron doses of 20, 40 and 80 mg/kg b.w. significantly inhibited the cellular DNA damage induced by the antitumor drugs, suggesting that saffron could be an adjuvant in chemotherapeutic applications (Premkumar *et al.* 2006).

1. Skin cancer

Skin carcinogenesis is a malignant growth of the epidermis that could be caused by UV-A and UV-B- radiation that generate free radicals in the cells. It was found that a saffron infusion (200 mg/kg b.w./d) has a beneficial action when given before and after the induction of skin carcinogenesis. Saffron ingestion inhibited the formation of skin papillomas and simultaneously reduced their size, fact that at least in part, may be due to the induction of cellular defense systems (Das *et al.* 2010).

2. Pancreatic cancer

Pancreatic cancer accounts for a high mortality rate because it has a very poor prognosis, so new therapeutic alternatives are really needed. Given saffron antitumour activity *in vitro* and *in vivo*, the proliferation of pancreatic adenocarcinoma cells is significantly inhibited due to a crocetin treatment (4 mg/kg b.w. /d) in mice. Also, pancreatic cancer growth was also significantly inhibited because of crocetin oral treatment (Dhar *et al.* 2009).

3. Breast cancer

Crocus sativus and different types of *Crocus taxa,* endemic in Greece, containing hydrophilic carotenoids show a dose-dependent inhibitory effect on breast cancer cells proliferation, attributing this effect to crocin contents in saffron (Chryssanthi *et al.* 2007). Some authors (Bathaie *et al.* 2007; Kanakis *et al.* 2007a, 2007b) mentioned that saffron carotenoids interact with DNA and induce some conformational changes on it, having crocetin the most potential.

4. Lung cancer

This type of cancer is the leading cause of cancer related mortality worldwide; so many efforts have been done in order to reduce it. A treatment of 20 mg/kg b.w. of crocetin dissolved in dimethyl sulphoxide was administered in mice, resulting in a reversion of the pathological changes observed in cancerous animals proving the antitumor ability of this compound (Magesh *et al.* 2006).

5. Colorectal cancer

Saffron inhibition on three colorectal cancer cell lines (HCT-116, SW-480 and HT-29) was studied, finding a dose-dependent inhibition of malignant cells growth, being crocin the major responsible of this activity. Moreover, crocin did not affect normal cells growth (Aung *et al.* 2007).

Antinociceptive effects

The antinociceptive, a well known property of saffron, due to their content of flavonoids, tannins, anthocyanins, alkaloids and saponins which was confirmed using safranal at doses between 0.1 and 0.5 ml/kg b.w. (Hosseinzadeh and Shariaty 2007). However, the mechanism responsible remains to be investigated (Hosseinzadeh and Younesi 2002).

Premenstrual syndrome

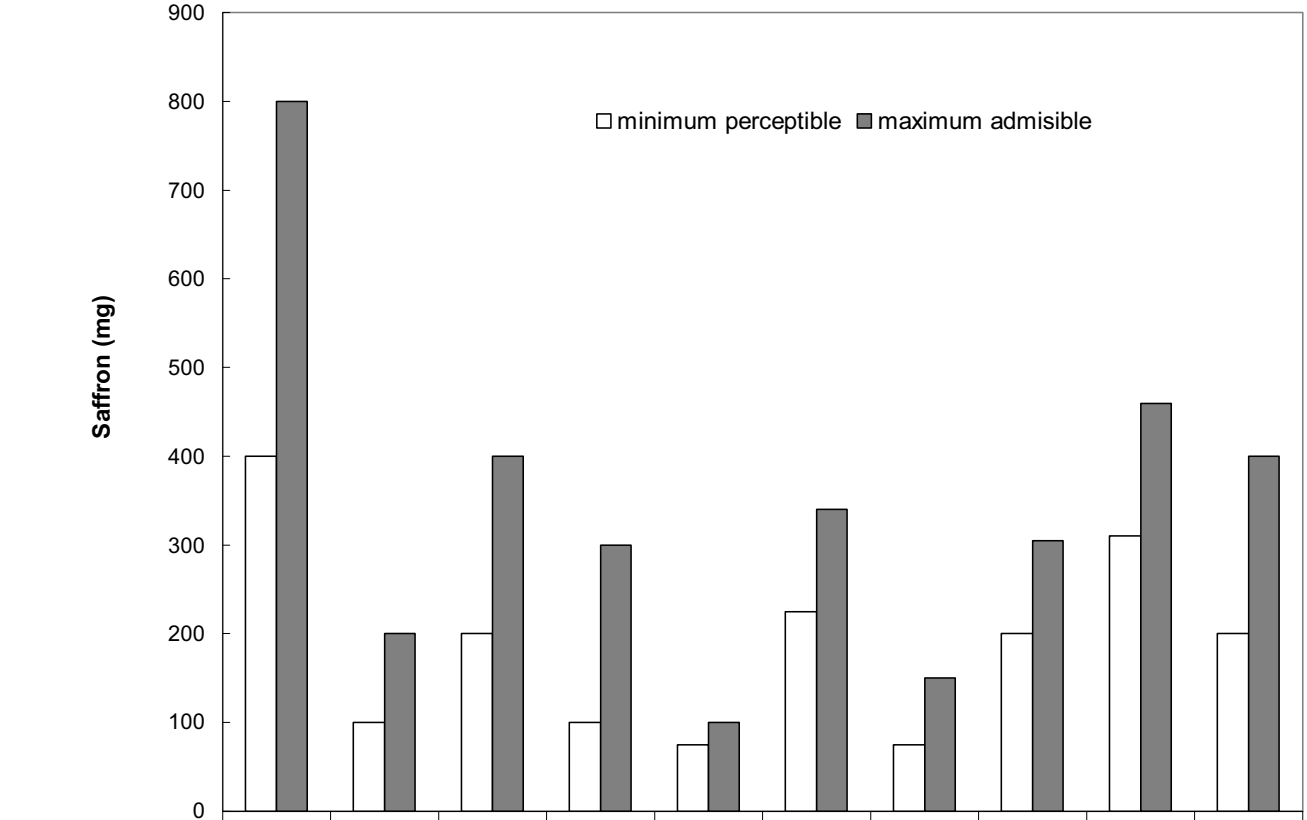
Premenstrual syndromes (PMS) are among the most common health problems reported by women of reproductive age characterised by emotional, behavioural and physical symptoms. There is an overlap between symptoms of depression and those associated with PMS, so saffron also resulted to be effective on treating this syndrome. Women between 20 and 45 years received 15 mg of saffron capsule twice a day, resulting in a relief of several symptoms. Even if the study is in line with previous reports, further research in this area is needed, because it is the first clinical trial done (Agha-Hosseini *et al.* 2008).

Sexual behaviour dysfunction and infertility

Sexual dysfunction is a serious medical and social symptom that occurs in 10-52% of men and 25-63% of women (Porst 2004). Since the available drugs and treatments for these problems have limited efficacy or side-effects, a series of plants, such as saffron, have been proved to have aphrodisiac effects. This fact is confirmed in a study using saffron extracts (80, 160 and 320 mg/kg b.w.) and crocin (100, 200 and 400 mg/kg b.w.), resulting an activity compared to sildenafil, an phosphodiesterase inhibitor, commonly used for treating erectile dysfunction, unlike safranal that showed a vasodilator effect (Hosseinzadeh *et al.* 2008). Recently, a pilot study was published, conducted with twenty male patients with erectile dysfunction, in which 200 mg of dried saffron stigma were taken orally during ten days every morning. After this period of time there was a statistically significant improvement on sexual function with increased number and duration of erectile events (Shamsa *et al.* 2009).

Table 1 Biomedical properties of saffron studied using patients.

Properties	References	Doses (saffron mg)	Frequency
Sexual behaviour dysfunction and infertility	Shamsa *et al.* 2008	200	Daily, 10 days
	Heidary *et al.* 2008	50	3 times a week, 12 weeks
Alzheimer	Akhondzadeh *et al.* 2010	30	Daily, 22 weeks
Depression	Akhondzadeh *et al.* 2004; Noorbala *et al.* 2004, 2005; Moshiri *et al.* 2006; Akhondzadeh Basti *et al.* 2007	30	Daily, 6-weeks
	Akhondzadeh Basti *et al.* 2008	30	Daily, 8-weeks
PMS	Agha-Hosseini *et al.* 2008	30	Daily, 8-weeks



	Oils	Fish soup	Meat soup	Mashed vegetables	Stew	Bread	Rice	Croissant	Pasta	Infussion
minimum perceptible	400	100	200	100	75	225	75	200	310	200
maximum admisible	800	200	400	300	100	340	150	305	460	400

Fig. 3 Minimum and maximum admissible saffron doses in different dishes (mg/L). Based on but modified from Verdú Cantó 2009.

Aside, the effects of saffron on men with idiopathic infertility was proved to be effective, based on an intake of 50 mg of saffron 3 times a week during 3 months, but further research are needed (Heidary *et al.* 2008).

Other studies

More biological applications of saffron and its constituents have been studied such as encephalomyelitis (Ghazavi *et al.* 2009), the hormone changes in pituitary-testis axis of mice (Nazem *et al.* 2009), possible fertility improvement (Ai *et al.* 2009), as a treatment for hemorrhagic shock (Yang R *et al.* 2006), the effects on the fetal development of mice (Golalipour *et al.* 2008), the efficacy against pneumonia (Mannan *et al.* 2006), pancreas-protective effects of saffron ethanolic extracts (Mohajeri *et al.* 2009), protective effects against nephrotoxicity (Boroushaki and Sadeghnia 2009), tyrosinase inhibitory activity (Li and Wu 2002), morphine dependence inhibition (Sahraei *et al.* 2008), among others, but further study needs to be done, in order to know more about the mechanisms of action of saffron and its constituents.

SAFFRON INTAKE AND HUMAN EQUIVALENT DOSES TRANSLATION

Many reviews have been published in the past recent years (Deng *et al.* 2002; Abdullaev and Espinosa-Aguirre 2004; Schmidt *et al.* 2007; Soeda *et al.* 2007; Kianbakht 2008) in order to synthesize saffron properties and its related research, but it is difficult to understand the human repercussion of these studies because most of them use animal doses that are not directly related neither to human doses nor saffron consumption. Studies using patients, principally Iranian, are very few, as presented in **Table 1**, treating problems related to sexual behaviour and depression, principally. For treating sexual behaviour, the studies used 50 and 200 mg during several weeks, in order to increase the number and duration of erectile events. In depression studies, they use unique doses, independently of the body weight of the patient, being 30 mg daily of saffron during 6 or 8 weeks the most common dose used for depression that cause an improvement.

The 30 mg dose per day can easily be attained eating saffron in different food dishes, as shown in **Fig. 3**, which presents minimum perceptible and maximum admissible doses of saffron (mg) per litre of different dishes such as soups, rice, pasta and pastry products, including a saffron

Table 2 K_m factors of different species for conversion of animal doses to human equivalent doses based on BSA.

Species	Weight (kg)	BSA (m^2)	K_m factor
Adult Human	60	1.6	37
Guinea pig	0.4	0.05	8
Rat	0.15	0.025	6
Mouse	0.02	0.007	3

FDA Draft Guidelines 2002

infusion. Most of them can be prepared using between 300 and 500 mg maximum of saffron per litre of food, being oil preparation the product that use more saffron, but its concentration is diluted taking into account that oil is used to prepare a wide variety of dishes and not eat alone. Saffron infusion represents a good way to increase saffron consumption because it is drink directly, without quantity restrictions. A cup of 100-150 ml can contain the active compounds corresponding to the mentioned dose of 30 mg of saffron per day.

In order to compare animal doses used in the majority of the studies with possible human doses, it is necessary to transform these quantities using the body surface area (BSA) normalization, because of converting the safe starting dose based on body weight alone, can result an inappropriate comparison between studies because of the lack of correlations for oxygen utilization, caloric expenditure, blood volume, circulating plasma proteins and renal functions between various mammalian species and differently sized members of the same species, including humans (Reagan-Shaw *et al.* 2007). In this study the recommendation of the U.S. Food and Drug Administration of using BSA normalization has been employed for the purpose to calculate the hypothetical human equivalent doses (HED), parameter used on initial clinical trials in healthy adult volunteers.

The customary approach for calculation of BSA uses the Du Bois height-weight formula: BSA (m2) equals body weight (kg b.w.)$^{0.425}$ multiplied by height (cm)$^{0.725}$ multiplied by 0.007184, has been re-evaluated in similar forms with updated constants, however scientific evidence does not favour one alternative formula over another (Sawyer

Table 3 Human equivalent doses calculated for the different saffron animal studies.

Effects	Reference	Animal	Saffron product	Frequency	Saffron equivalent doses (mg/kg b.w.)[a]	HED (mg/person)[b]
Biological activities						
Antioxidant	Hosseinzadeh *et al.* 2007b	Rats	Ethanolic extract	Mono dose	5 - 80	57 - 908
			Crocin		172 - 1379	1 957 - 15 657
			Safranal		14 505 - 72 523	164 646 - 823 229
Ulcers	Al-Mofleh *et al.* 2006	Rats	Extract	Mono dose	250	2 838
	Kianbakht and Mozaffari 2009	Rats	Extract		25 - 250	284 - 2 838
			Crocin		8 - 35	88 - 391
			Safranal		362 613 - 725	4 116 144 - 8 232
					225	286
Nervous system damage						
Neuronal injury	Hosseinzadeh and Sadeghnia 2005	Mice	Safranal	Mono dose	109 234	1 239 956
Retinal function	Maccarone *et al.* 2008	Rats	Extract	Mono dose	1	11
Parkinson	Ahmad M *et al.* 2005	Rats	Crocin	Daily, 7 days	0.3	3
Seizures	Hosseinzadeh and Khosravan 2002	Mice	Ethanolic extract	Mono dose	0.2 - 2	1 - 11
			Aqueous extract		0.1 - 0.8	0.45–5
	Hosseinzadeh and Talebzadeh 2005	Mice	Safranal		21 757 - 50 766	123 484 - 288 130
	Hosseinzadeh and Sadeghnia 2007	Rats	Safranal		10 923 - 43 694	123 996 - 495 982
Learning behaviour	Pitsikas and Sakellaridis 2006	Rats	Extract	Mono dose	30 - 60	341 - 681
	Pitsikas *et al.* 2007	Rats	Crocin	Daily	52 - 103	587 - 1 174
Anxiety	Pitsikas *et al.* 2008	Rats	Crocin	Mono dose	172	1 957
	Hosseinzadeh and Noraei 2009	Mice	Extract		56 - 560	318 - 3 178
			Safranal		21757 - 50 766	123 484 - 288 130
Sedative/relaxant	Hosseinzadeh and Ghenaati 2006	Guinea pigs	Ethanolic extract	Mono dose	100 - 800	1 514 - 12 108
			Safranal		36 261 - 108 784	548 819 - 1 646 457
Depression	Karimi *et al.* 2001	Mice	Safranal	Mono dose	23 - 75	128 - 426
			Crocin		172 - 2 079	979 - 11 743
	Hosseinzadeh *et al.* 2004	Mice	Aqueous extract		160 - 320	908 - 1 816
			Ethanolic extract		200 - 800	1 135 - 4 541
Cardiovascular injury						
Atherosclerosis	Sheng *et al.* 2006	Rats	Crocin	Daily, 10 days	86 - 1 250	979 - 14 189
	Asqad *et al.* 2009	Rats	Extract	Daily, 5 days	25 - 100	284 - 1 135
Myocardial infarction	Goyal *et al.* 2009	Rats	Crocin	Daily, 21 days	69	783
	Yan *et al.* 2010	Rats	Crocin		172	1957
Pheripheral vascular diseases	Yang *et al.* 2008	Rats	Crocin	Daily, 2 days	86 - 625	979 - 7 095
Insulin resistance	Xi *et al.* 2007	Rats	Crocin	Daily, 8 weeks	69 - 138	783 - 1 566
Cancer and tumours						
Cancer and tumours	Premkumar *et al.* 2006	Mice	Extract	Daily, 5 days	20 - 80	114 - 454
Skin cancer	Das *et al.* 2009	Mice	Extract	Daily, 7 days	200	1 135
Pancreatic cancer	Dhar *et al.* 2009	Mice	Crocin	Daily, 30 days	14	78
Lung cancer	Magesh *et al.* 2006	Mice	Crocin	Daily, 4 weeks	69	391
Antinociceptive effects						
Antinociceptive	Hosseinzadeh and Shariaty 2007	Mice	Safranal	Mono dose	14 505 - 72 522	82 323 - 411 614
Sexual behaviour dysfunction						
Sexual behaviour dysfunction	Hosseinzadeh *et al.* 2008	Rats	Extract	Mono dose	80 - 320	908 - 3 632

[a] Doses were converted to mg/kg b.w. of saffron equivalent, taking into account a saffron humidity of 9%, 0.66% safranal content and 32% on dry basis of crocetin content
[b] HED were calculated using K_m factors based on BSA. The final HED was multiplied by a body weight of 70 kg

and Ratain 2001; Wang and Hihara 2004; Verbraeclen *et al.* 2006; Reagan-Shaw *et al*. 2007). BSA is often represented in mg/m2 and can be translated to human equivalent doses (HED) in mg/kg b.w. according to this formula: HED (mg/kg) equals to animal dose (mg/kg) multiplied by animal Km/ human Km (Reagan-Shaw *et al*. 2007) using factors named K_m factors, for the different species summarized on **Table 2**.

This study pretends to calculate a tentative HED from the animal doses of the different saffron studies and link it to saffron consumption in different dishes. This work it is a first attempt to suggest a tentative reference for human doses that can not be taken lightly, because of the lack of pharmacokinetics studies in the bibliography and other very important data such as LD_{10} values, bioavailability, absorption and elimination kinetics of the saffron compounds in humans. Human equivalent doses for the different saffron properties are shown in **Table 3**, calculations are based on the range of doses proved on each animal study that did not cause toxicity and exerted a noticeable effect. Doses were converted to mg/kg b.w. of saffron equivalent, taking into account an average saffron moisture of 9% (Carmona *et al.* 2006), up to 0.66% safranal content (Maggi *et al.* 2009) and up to 32% crocetin ester content (Sánchez *et al.* 2009). Differences between aqueous and ethanolic extracts were not considered. The final human dose was multiply by a body weight of 70 kg.

From **Table 3**, it can be observed that some of these doses are really approachable for adults, such as antioxidant activity (57-908 mg), depression (128-426 mg) and learning behaviour (341-681), being seizures (1-11 mg) and Parkinson the diseases that needs less saffron doses for its prevention or amelioration. In the other hand, studies which used safranal, show doses that can not be achieved by just eating dishes with saffron, taking into account that safranal content in saffron represents a 0.66% of its composition and in the studies the majority of safranal source was a standard of high purity.

Saffron intake in food, as shown by data presented in this work, could represent a good way to achieve different biological effects and taken daily as a habitude, could represent a preventing method for many diseases, specially taken as an infusion.

CONCLUSION

Saffron has been investigated during years in a wide variety of different biological effects. A big part of these effects are achieved by its antioxidant properties, since it is responsible for many chemical reactions that have effects on preventing many diseases, such cardiovascular and neuronal injury, among others. It is recommended further investigation and clinical trials because of deficiencies on some studies conducted and the lack of pharmacokinetics studies in order to have a better correlation between animal and human doses. Saffron consumption in food could represent a good source for preventing many diseases. Doses of clinical trials made in human patients can be achieved by consuming saffron in food, especially as an infusion.

ACKNOWLEDGEMENTS

We thank the company Verdú Cantó Saffron Spain S.L. for supplying the valuable data about the maximum and minimum admissible doses of saffron in food.

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Saffron and Other Spices as Potential Allergenic Sources

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ABSTRACT

Spices are used as food additives to confer flavour, odour and colour or as preservatives that kill microorganisms or prevent their growth. Many of these substances can also produce allergic reactions with symptoms that range from mild and local to severe systemic disorders. Spice allergies are a growing problem, with strict avoidance of allergens as the only effective treatment. Since spices are extensively consumed in homemade meals and also contained as hidden condiments in many pre-packaged foods, adverse reactions are often difficult to prevent. This article is an overview of the most important spices responsible for immediate hypersensitivity reactions mediated by IgE, with special attention given to saffron, the world's most expensive spice.

Keywords: advisory labelling, allergen, cross-reactivity, saffron, spice allergy
Abbreviations: Art v, *Artemisia vulgaris*; **Bet v 1**, PR10 birch pollen allergen; **Bet v 2**, birch pollen profilin; **Bra j 1**, brown mustard 2S albumin; **Bra o 3**, cabbage LTP; **Cro s 2**, saffron profilin; **Cuc m 4**, melon profilin; **rCro s 3.01**, recombinant saffron lipid transfer protein 1; **rCro s 3.02**, recombinant saffron lipid transfer protein 2; **LTP**, lipid transfer protein; **PRs**, pathogenesis-related proteins; **Pru p 3**, peach lipid transfer protein; **Sal k 1**, major *Salsola kali* pollen allergen; **Sin a 1**, yellow mustard 2S albumin; **Sin a 3**, *Sinapsis alba* lipid transfer protein; **Sin a 4**, *Sinapsis alba* profilin; **SLIT**, sublingual immunotherapy

CONTENTS

INTRODUCTION

Allergies are one of the illnesses that have increased on a worldwide scale over the past ten years, particularly in developed countries (Sampson 2005; Takeda and Gelfant 2009). It is expected that by 2020 over 50% of the population in westernized countries will suffer from allergies. An allergic reaction can be caused by any form of contact with allergens, i.e. by ingestion (eating or drinking), inhalation (pollen, house mites, etc.) or direct contact. In most cases, the agent responsible for this reaction is a protein that activates mast cells and basophils by means of an immunoglobulin known as IgE, resulting in an extreme inflammatory response. Hypersensitivity mediated by IgE is the result of mast cells and basophil mediators. Clinical symptoms are a result of cross-linking of IgE and aggregation of high-affinity receptors for IgE on mast cells and basophils. On activation, mast cells and basophils release both preformed mediators such as histamine and tryptase and newly formed ones such as leukotrienes and prostaglandins. These mechanisms allow recruitment of eosinophils, monocytes and lymphocytes in the area affected in the late phase response and release a variety of cytokines and inflammatory responses (Metcalfe and Peavy 2009; Simons 2009) (**Fig. 1**). This reaction is only produced in individuals with a predisposition for developing allergies. The typical symptoms of allergic reactions can occur on the skin in the form of hives, eczema, itching or swelling; in the gastrointestinal tract,

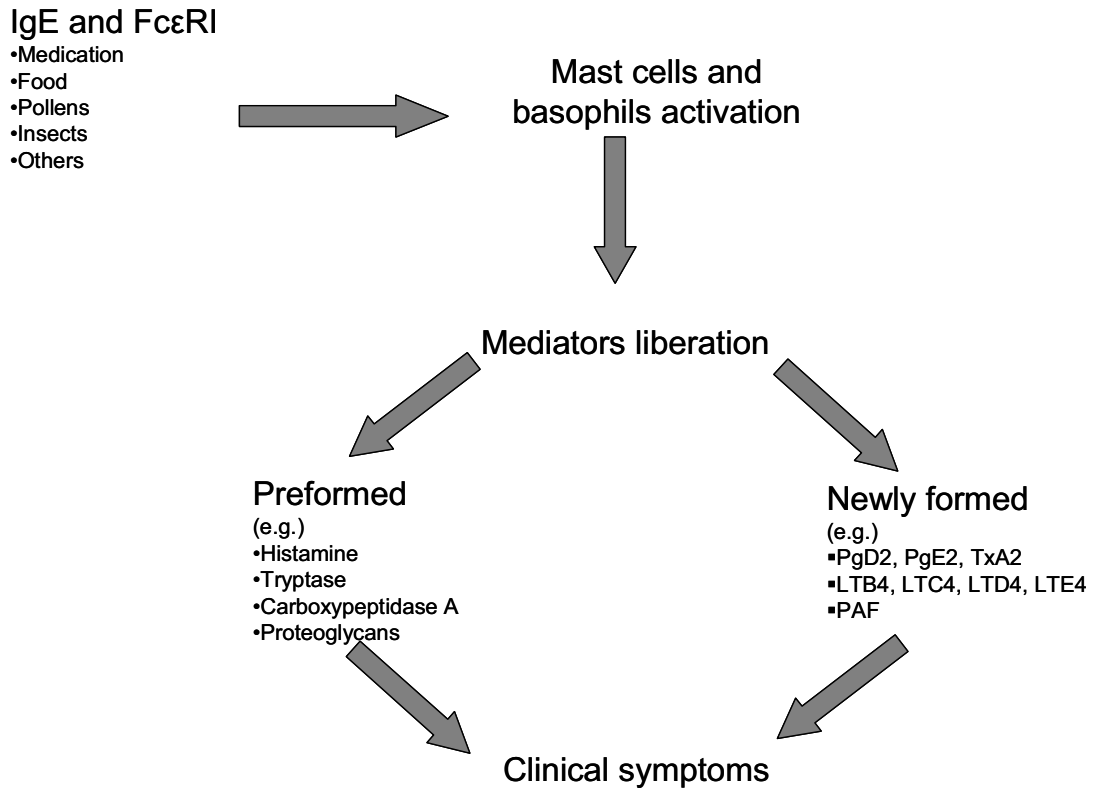

Fig. 1 Mechanisms of IgE-mediated allergenic reaction. Clinical symptoms are a result of cross-linking of IgE and aggregation of high-affinity receptors for IgE on mast cells and basophils. Common triggers include foods, medications, insect stings, and pollens. On activation, mast cells and basophils release both preformed and newly formed chemical mediators, only a few of which are listed.

producing nausea, vomiting or diarrhea; and in the respiratory tract as asthma, rhinitis, laryngeal edema or throat swelling. In highly sensitive individuals, ingestion of specific foods can produce anaphylactic shock which may even lead to death if appropriate treatment is not given (Tang *et al.* 2009).

FOOD ALLERGY

Food allergies affect 6% of young children and 3-4% of adults in westernized countries (Pieretti *et al.* 2009; Sicherer and Sampson 2009). There is no treatment for food allergies so specialists recommend strict avoidance of offending food allergens. However, numerous strategies for definitive treatment are being investigated, including sublingual/oral immunotherapy, injection of anti-IgE antibodies, etc. (Sicherer and Sampson 2009). For instance, Fernandez-Rivas *et al.* (2009) have recently shown the efficacy and safety of sublingual immunotherapy (SLIT) with a peach extract quantified in mass units for Pru p 3, the peach lipid transfer protein. They point out that the SLIT for peach allergy could be a promising therapeutic option that may modify patients' clinical reactivity to peach intake.

Food allergens are divided into two classes. Class 1 food allergens are also called true food allergens. This kind of allergen is almost always a protein which elicits both the sensitization and the effector phases of the IgE-mediated food allergy. Class 2 food allergens provoke allergic reactions in patients previously sensitized by inhalation or contact with homologous allergens from other allergenic sources such as pollen (Egger *et al.* 2006). Allergens belonging to Class 1 seem to share common characteristics, e.g. resistance to digestive enzymes and heat, thus indicating that sensitization occurs in the gastrointestinal tract. Class 2 food allergens are in general more sensitive to gastric digestion and heat and appear to cause a mild oral reaction.

A common feature in patients allergic to a particular agent is their awareness of other allergenic sources. Cross reactions have been reported among similar agents in closely related species, such as birch and hazel pollen (Valenta *et al.* 1991) or fruits of the same botanical family (Pastorello *et al.* 1994; Ahrazem *et al.* 2005), and also between phylogenetically distant species, such as birch pollen and apple fruit or latex and some fruits (Salcedo *et al.* 2001; Hofmann and Burks 2008; Bartra *et al.* 2009). Cross-reactivity is caused by the presence of Ig epitopes within the proteins involved in allergies. For example, the distribution of members of the Bet v 1 family (the main allergen of birch pollen) in different pollens, fruits and vegetables is well documented (Vieths 1997; Breiteneder and Ebner 2000; Breiteneder and Radauer 2004); also profilins are cross-reactive plant allergens responsible for multiple pollen sensitization and pollen-associated food allergy (Rauder *et al.* 2006; Bonds *et al.* 2008). Many identified food allergens are also widely distributed throughout the plant kingdom and are involved in the cross-reactions between antigens from unrelated plant species, therefore being recognized as panallergens.

The majority of plant food allergens can be grouped into just 4 protein families: 1) the prolamin superfamily containing several allergenic (2S albumins, nonspecific lipid transfer proteins, cereal amylase and protease inhibitors) and prolamins member families; 2) the cupin superfamily comprising numerous functionally highly diverse protein families, although allergenicity within the cupins is observed mainly in the vicilin and legumin seed storage proteins; 3) the profilin family which contains ubiquitous eukaryotic proteins that are nonallergenic, with the exception of profilins from flowering plants; and 4) the Bet v 1 superfamily containing various families, with the pathogenesis-related protein 10 family as the only allergenic members (Radauer and Breiteneder 2007). When the amino acid sequences of all known food allergens are analyzed and compared, the majority of them can be included in some of the 17 described pathogenesis-related protein (PR) families (Breiteneder and Ebner 2000). Most PRs and related proteins are induced through the action of the signalling compounds such as salicylic acid, jasmonic acid, or ethylene, and possess antimicrobial or insecticidal activities (van Loon *et al.* 2006). Examples of allergens homologous to PRs are summarized in **Table 1**.

Table 1 Major pathogenesis-related proteins (PR) families involved in plant food allergy.

PR family/species/common name	Representative allergens	Protein classification
PR10		Bet v 1 homologous proteins
Malus domestica/Apple	Mal d 1	
Pyrus communis/Pear	Pyr c 1	
Prunus avium/Cherry	Pru av 1	
PR14		Lipid transfer protein
Prunus persica/Peach	Pru p 3	
Malus domestica/Apple	Mal d 3	
Coryllus aveana/Hazlnut	Cor a 8	
PR5		Thaumatin-like protein
Prunus avium/Cherry	Pru av 2	
Actinidia deliciosa/Kiwi	Act d 2	
Malus domestica/Apple	Mal d 2	
PR3		Class I chitinases
Persea americana/Avocado	Pers a 1	
Musa acuminata/Banana	Mus a 2	
Castanea sativa/Chesnut		
PR2		β-1,3-Glucanases
Musa acuminata/Banana	Mus a 5	

Spices are commonly used in cooking in order to add flavour, odour and visual appeal to food. They are usually made from seeds, bark, roots, fruits, or flowers of plants, and they are generally used as dried products with a brown, black or red colour and a pungent smell (Lai and Roy 2004). According to the United States Code of Federal Regulations, most spices are recognized as safe for human consumption, although spices considered toxic may provoke allergic reactions, ranging from mild and local to severe systemic. They can enter the body through inhalation or by ingestion even after prolonged cooking, thus suggesting the existence of resistant allergens. The following sections present data on allergies to the most important spices with specific reference to saffron.

ALLERGY TO SAFFRON

Saffron is a spice derived from the dry stigmas of the *Crocus sativus* flower. It is used in cooking as a seasoning and colouring agent and considered to be the most expensive spice in the world.

During the last ten years, numerous studies focusing on the biological and pharmaceutical properties of saffron have shown its implication in the reduction of cholesterol and triglyceride levels in the blood (Abdullaev *et al.* 1999), its capacity to combat neural disorders (Abe *et al.* 2000), as well as its role as an attenuator of the adverse effects of cisplatin used in chemotherapy (Premkumar *et al.* 2003). Saffron is traditionally used for medicinal purposes as a stimulant; aphrodisiac and antidepressant, while evidence of antitumoural effects in various cellular models is also present (Schmidt *et al.* 2007). Saffron has three main pharmacologically active metabolites: crocins, the water-soluble carotenoids that give saffron its colour; pirocrocin, responsible for the bitter taste; and safranal, the volatile compound that provides odour and aroma (Lai and Roy 2004; Moraga *et al.* 2009).

The planting of saffron corms is a difficult task with bulbs being planted one by one and by hand. Harvesting and removal of saffron stigmas is also done manually. The conditions of cultivation and handling of saffron facilitate the development of allergies by inhalation (pollinosis, asthma) and by contact.

Research on saffron allergies is scarce. One relevant article (Feo *et al.* 1997) showed the implication of saffron flowers in allergy development and its clinical significance as an occupational allergy. These authors analysed the IgE-binding fractions of saffron pollen and saffron stamens in 13 sera from sensitized patients. Their studies revealed the presence of a profilin with a molecular weight of 15.5 kDa showing a high IgE reactivity in all the sera.

Saffron allergy can even produce anaphylaxis. Wuthrich *et al.* (1997) reported the case of a German farmer who experienced a severe anaphylactic reaction after consuming a saffron rice dish. This reaction was due to high molecular weight proteins (40-90 kDa) present in the saffron extract.

Martínez *et al.* (2007) described the case of a grower with occupational airborne contact dermatitis caused by saffron bulbs. The grower showed eczematous and erythematous lesions on the back of his hands, forearms, neck, face and inframammary region, all associated with cleaning saffron corms previous to planting. These authors discarded the implication of the alpha-methyl-gamma-butyrolactone, which can cause similar symptoms in tulip bulb sensitivity, as the agent responsible for saffron allergy.

Varasteh *et al.* (2007) selected thirty-eight subjects with clinical manifestations of saffron pollen allergy in the nose (sneezing, blockage, and running), eyes (itching, redness, tears, and swelling), mouth and throat (itching and dryness), and chest (breathlessness, cough, wheezing, and tightness) to study saffron pollen allergenicity. In this study, 70% of the subjects showed an immediate reaction to saffron extract. These authors showed the involvement of saffron pollen as an aeroallergen by using skin prick tests with saffron extract in atopic subjects. Sankian *et al.* (2008) purified a profilin from saffron pollen named Cro s 2 involved in occupational allergy. In 2009, Varasteh *et al.* (2009) cloned the saffron profilin, expressed the recombinant rCro s 2 and showed its cross-reactivity with other plant profilins.

Saffron profilin Cro s 2 shared 73% of sequence identity with melon profilin Cuc m 2, but several non-conservative amino acid changes were detected in the regions corresponding to the weak and strong IgE epitopes defined for the melon allergen by López-Torrejón *et al.* (2007).

Recently, Gómez-Gómez *et al.* (2010) studied the involvement of lipid transfer proteins in saffron allergy. They selected six patients on the basis of clinical symptoms suggestive of a type I hypersensitivity reaction to saffron and with positive skin prick test responses and specific IgE to saffron extract. Immunodetection of saffron extract was performed with a rabbit polyclonal antiserum against the peach lipid transfer protein (Pru p 3), as well as with sera from saffron-allergic patients, revealing a band with an apparent molecular weight of approximately 9 kDa. Two LTPs, named rCro s 3.01 and rCro s 3.02 (Accession nº FJ997554, and nº FJ997555), have been isolated and expressed in *Pichia pastoris*. The authors pointed out that rCro s 3.01 and rCro s 3.02 were minor saffron allergens, with this report being the first on the involvement of LTPs in spice allergy. In addition, two allergenic members of the LTP family with a limited amino acid sequence identity (under 50%) have been found in a single plant source. Interestingly, ELISA tests showed cross-reactivity between Pru p 3 and the LTPs isolated from saffron.

Table 2 List of spices allergens included in the Official List of Allergens (International Union of Immunological Societies) updated in 27-01-2010. N.A.: Not available.

Species	Common name	Allergen	Biochemical name	Molecular weight (SDS-PAGE) (kDa)
Apium graveolens	Celery	Api g 1	Pathogenesis-related protein, PR-10	9
		Api g 2	Non specific lipid-transfer protein, type 1	15
		Api g 3	Chlorophyll *a-b* binding protein, chloroplast	N.A.
		Api g 4	Profilin	14
		Api g 5	FAD-containing oxidase	52
Brassica juncea	Oriental mustard	Bra j 1	2S seed storage albumin	N.A.
Sinapsis alba	Yellow mustard	Sin a 1	2S albumin	14
		Sin a 2	11S albumin	51
		Sin a 3	Non specific lipid-transfer protein	12
		Sin a 4	Profilin	14
Capsicum annum	Bell pepper	Cap a 1w	Thaumatin-like protein	23
		Cap a 2	Profilin	14
Sesamum indicum	Sesame	Ses i 1	2S Albumin	9
		Ses i 2	2S Albumin	7
		Ses i 3	7S Vicilin-like globulin	47
		Ses i 4	Oleosin	17
		Ses i 5	Oleosin	15
		Ses i 6	11S Globulin	52
		Ses i 7	11S Globulin	57

ALLERGY TO OTHER IMPORTANT SPICES

Mustard

Mustard, which belongs to the *Brassicaceae* family, includes three types of seeds: yellow (*Sinapsis alba*), brown (*Brassica juncea*) and black (*Brassica nigra*). Black seeds constitute one of the most frequent causes of allergy although they are not widely consumed due to difficulties in harvesting (Uhl 2000). Several reactions to this spice in the form of an immediate skin reaction, angioedema, and anaphylactic shocks have been reported in patients after ingestion of mustard seed flour or manufactured foods (Panconesi *et al.* 1980; Widstrom and Johansson 1986; Jorro *et al.* 1995; Kanny *et al.* 1995; Palomares *et al.* 2007; Sirvent *et al.* 2009). Major allergens of yellow and brown mustard seeds, Sin a 1 and Bra j 1, respectively (**Table 2**), have been characterized as seed storage proteins, belonging to the 2S albumin family, with an approximate molecular weight of 14 to 16 kDa (Menéndez-Arias *et al.* 1988; Gonzalez de la Peña *et al.* 1991). In 2007, an 11S globulin storage protein of 51 kDa was isolated and identified as a novel major allergen of yellow mustard seeds (Palomares *et al.* 2007). More recently, two allergens, Sin a 3 and Sin a 4, corresponding to a non-specific lipid transfer protein and a profiling, respectively, have been isolated. These proteins were IgE-reactive in ELISA and immunoblotting and showed IgE cross-reactivity with fruits such as peach (Pru p 3) and melon (Cuc m 4) (Sirvent *et al.* 2009). Furthermore, Figueroa *et al.* (2005) have proved mustard allergy by double-blind placebo-controlled food challenges. Mustard has been recently included in the list of 12 potential allergenic foods to be labelled in a European Union directive on the identification of foods (Pieretti *et al.* 2009).

Apiaceae spices: anise, fennel, coriander and cumin

Jensen-Jarolim *et al.* (1997) investigated allergens originating from anise (*Pimpinella anisum*), fennel (*Foeniculum vulgare*), coriander (*Coriandrum sativum*) and cumin (*Cuminum cyminum*) spices in 15 patients who showed adverse reaction to spiced food. These patients showed the presence of Bet v 1 homologous in all the spices and the presence of profilin in anise and cumin extracts. High molecular weight proteins binding IgE were also detected. ELISA assays confirmed the role of profilin and Bet v 1. The authors pointed out that only patients with birch and/or mugwort pollinosis and/or celeriac allergy showed positive reactions to the tested spices. These data reinforced the frequent coincidence of the mugwort-celery-syndrome with food allergy to spices (Wuthrich *et al.* 1990; Jensen-Jarolim *et al.* 1997; Egger *et al.* 2006).

Pepper and paprika

Pepper (*Piper nigrum*) and paprika (*Capsicum annum*), belonging to the *Piperaceae* and *Solanaceae* families, respectively, are widely used as spices in international cuisine. Wultrich (1993) studied 402 patients from Central Europe with food allergies, 5.7% of which had allergies to paprika and 1% to pepper. Leitner *et al.* (1998) characterized allergens from these spices in 22 patients suffering from celery-mugwort-spice syndrome. In immunoblotting, 73% and 95% of patients displayed IgE to pepper and paprika proteins, respectively. Two major allergens (of 14 and 28 kDa) were found in pepper and one in paprika (23 kDa). The partial sequence of the 14 kDa pepper allergen did not permit the identification of similarities with others contained in the database banks, while the 28 kDa was a germin-like protein and the 24 kDa paprika allergen was a thaumatin-like protein.

Fenugreek

Fenugreek (*Trigonella foenum-graecum*) is a legume plant with maple-like flavour traditionally used as an ingredient of curry spice mainly added as an aromatic condiment to different manufactured foods. Incidents of IgE mediated allergy to fenugreek have been reported and a cross-reaction with allergy to peanut has been suggested. In 2010, Fæste *et al.* characterized allergens from fenugreek using mass spectroscopy based proteomic strategy. A reactive protein at 50 kDa has been described as the most consistent allergen in all the patients included in this work. The major allergen was characterized as a 7S vicilin-like protein named Tri f 1. Other allergens as pathogenesis-related proteins 10 (Tri f4), 2S albumin (Tri f 2) and 11S legumin (Tri f 3) have been also characterized. These allergens revealed high identities with Ara h 1, Ara h2, Ara h 3, Ara h 4, Ara h 6, Ara h 7 and Ara h 8 isolated from peanut. The authors concluded that significant homologies to major peanut allergens provide an explanation to the proliferation of secondary fenugreek allergy by cross-reaction in peanut allergic patients.

Sesame

Sesame (*Sesamum indicum*) seeds are used for the preparation of a range of traditional dishes and as flavouring agents in the preparation of specialty breads, cakes, and delicacies.

Other common foods containing sesame seeds include vegetarian burgers, spice mixtures, salad dressings and a wide range of diet foods. Sesame oil is also used in pharmaceutical products and the cosmetic industry (Orruño and Morgan 2006). Severe reactions to sesame, including anaphylactic shock, have been reported in the literature (Asero *et al.* 1999; Pajno *et al.* 2000; Dalal *et al.* 2003). Sesame hypersensitivity has been reported both in adult and pediatric populations (Sporik and Hill 1996; Rance *et al.* 1997; Levi and Danon 2001; Dalal *et al.* 2002). Both seeds and oil have been linked to allergic reactions (Chiu and Haydik 1991; Kanny *et al.* 1996; Morisset *et al.* 2003). Hypersensitivity reactions to products containing sesame oil have been described, constituting a risk for atopic patients (Pecquet *et al.* 1998). In 2001, Pastorello *et al.* identified a major sesame seed allergen named ses i 1 and was a 2S albumin with a molecular weight at 9 kDa. Later, Beyer *et al.* (2002) using sera from 20 patients with sesame seed allergy identified 4 sesame seed allergens a protein at 45 kDa, which was recognized by 75% of the patients, was found to be a 7S vicilin-type globulin, a seed storage protein of sesame and named Ses i 3. The protein at 7 kDa was found to be a 2S albumin, another seed storage protein of sesame and named Ses i 2. In addition, the proteins at 78 and 34 kDa were found to be homologous to the embryonic abundant protein and the seed maturation protein of soybeans, respectively. More recently, Leduc *et al.* (2006) showed the implication of two Oleosins named Ses i 4 (17 kDa) and Ses i 5 (15 kDa) in sesame seed allergy.

POLLEN-SPICE ASSOCIATIONS

In general, spice allergy results from cross-reactivity with pollen allergens such as mugwort (*Artemisia vulgaris*, Compositae) and birch (*Betula verrucosa*, Fagales) pollinosis. The resulting cross-allergies are summarized in the two syndromes listed below (Scholl and Jensen-Jarolim 2004; Egger *et al.* 2006).

Celery-mugwort-spice syndrome

This syndrome has been established to describe cross-reactivity between *Artemisia* and *Apiaceae* food (Egger *et al.* 2006). Ten different food allergies are associated to the Celery-mugwort-spice syndrome including *Apiaceae* (e.g. fennel (*Foeniculum vulgare*) and coriander (*Coriandrum sativum*)) and *Liliaceae* (e.g. garlic (*Allium sativum*) and onion (*Allium cepa*)). There is an evident association between birch, mugwort pollinosis and celery hypersensitivity, since these syndromes share common allergens. Patients with allergy to spices are young adults sensitized to mugwort and birch pollen, sharing cross-sensitization with various plants.

Mugwort-mustard allergy syndrome

Caballero *et al.* (2002) researched patients allergic to mustard who suffered from associated pollinosis or allergy to other plant-derived foods. Upon ingestion of mustard, patients showed diverse symptoms ranging from oral allergy syndrome to systemic anaphylaxis. More than 97% of the patients studied were allergic to mugwort, and 100% were sensitized to *Brassicaceae* plants. Using these data, Figueroa *et al.* (2005) suggested the term mugwort-mustard allergy syndrome to describe the association of mustard allergy with mugwort pollinosis and other plant-derived food allergies. Three possible allergens were suggested by Egger *et al.* (2006) that can be involved in this syndrome: Art v 60 kDa, profilin, and the mugwort nonspecific lipid transfer protein (Art v 3). In 2007, Palacin *et al.* searched for potential cross-reacting allergens widely distributed throughout the *Brassicaceae* family, using cabbage leaves (*Brassica oleracea* var *capitata*) as the target food. This approach enabled the authors to detect an LTP, named Bra o 3, as responsible for cross-reactions among *Brassicaceae* foods including mustard. They suggest that the LTP may play a crucial role in the mugwort pollen–mustard allergy syndrome.

OTHER POSSIBLE ASSOCIATIONS

Russian thistle-saffron association

Russian thistle (*Salsola kali*), which belongs to the *Chenopodiaceae* family, is considered the main cause of pollen allergy in arid and semiarid countries. In a recent study describing the sensitization profiles in complex pollen areas in Spain (Barber *et al.* 2008), *Salsola* was found to be the third most frequent cause of pollinosis in the southern part of the country. Sal k 1, a major allergen of *Salsola* (Barderas *et al.* 2007), is practically absent in other *Chenopodiaceae* species. The increase in *Salsola* allergy prevalence seems to be linked to global warming. In semiarid south-eastern areas of Spain, *Salsola* was the most frequent cause of seasonal allergies. An association between *Salsola* and saffron was suggested in a study on occupational allergies in Spanish saffron workers from Ciudad Real (Central Spain), where the climate is semiarid (Feo *et al.* 1997). However, the clinical significance of this finding remains to be elucidated.

FOOD ALLERGY LABELLING AND CONSUMER PROTECTION

Consumers with food allergy occasionally experience allergic reactions from the ingestion of food products containing undeclared ingredients derived from allergenic sources. Hefle *et al.* (2007) researched food products (n = 625 in 2003 and n = 645 in 2006) with advisory labels that listed peanut allergens and whether consumers with allergies ignored these advisory labels or not. The conclusions of this study stated that peanut allergens were detected in 10% of total products and that the percentage of consumers paying attention to advisory labels decreased to 75% in 2006, down from 85% in 2003. Pieretti *et al.* (2009) determined the frequency and language used in 20,241 manufactured food products, and identified labelling ambiguities affecting consumers with food allergy. This study showed that nonspecific terms, such as “spices”, were found on 65% of products and they were not labelled as specific ingredients in 83% of the cases. Allergists should advise their patients with spice allergy to avoid ingestion of packaged food products with ingredients listed as spices or natural flavour. Consumers with food allergy should pay attention to allergy advisory labelling and avoid consumption if they have any doubt about the risk of suffering an adverse reaction. It is also strongly recommended to food manufacturers to make the advisory labels clear and truthful, and not misleading.

CONCLUSIONS

The food industry, especially fast foods and exotic cuisines, have contributed to the spread of spice allergens that can be described as emerging. Anaphylactic recurrences are often associated with the presence of hidden ingredients such as spices and, much more rarely, additives. Among the frequently masked spice allergens, the most common are celery, coriander, anise, cumin, sesame, mustard and saffron. Despite their low incidence, spice allergies can provoke serious problems. Food labels should therefore alert individuals as to hidden ingredients, and a standardized list of allergens would be helpful to many consumers. Patients with food allergies should read the ingredient labels of packaged foods carefully, and allergists must advise their patients of this problem.

ACKNOWLEDGEMENTS

We are grateful for support from the “Junta de Comunidades de Castilla-La Mancha, Fundación de Investigación Sanitaria en C-LM” (FISCAM, PI-2007/61). We wish to thank KA Walsh (Escu-

ela Técnica Superior de Ingenieros Agrónomos, Universidad de Castilla-La Mancha, Albacete, Spain) for language revision. O Ahrazem received funding from the Spanish MICINN through the Ramon y Cajal Programme.

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Crocus sativus Pathogens and Defence Responses

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ABSTRACT

Saffron plants in their natural environment are constantly under siege by a multitude of disease-causing organisms including bacteria, fungi, viruses and nematodes. These phytopathogens invade into the plant apoplast and proliferate by assimilating nutrients from plant cells, hence provoking important economic damage to saffron around the world. Most pathogenic species affect the corm, causing pre- and post-development of this organ, which in turn affects saffron viability, propagation and yield. However, only a relatively small proportion of these pathogens is capable of invading the host plant successfully and causing disease. Plants depend on sophisticated defensive strategies to resist this invasion, using both preformed and inducible defence responses. This ability to resist disease also depends on soil conditions such as structure, compaction, drainage, temperature and level of biological activity, along with farming practices that influence plant development, such as planting date and application of fertilisers or herbicides. Our ability to exert sustainable control over saffron diseases relies on a two-fold understanding of saffron development and defence mechanisms.

Keywords: bacteria, *Crocus*, fungi, genes, nematodes, transposable elements, virus
Abbreviations: BYMV, *Bean yellow mosaic virus*; **CMV**, *Cucumber mosaic virus*; **CW**, cell wall; **EST**, expressed sequence tag; **HR**, hypersensitive response; **ISMV**, *Iris severe mosaic virus*; **NMV** *Narcissus mosaic virus*; **PAMP**, pathogen-associated molecular pattern; **PCR**, polymerase chain reaction; **PR**, pathogenesis related; **RT-PCR**, reverse transcription-polymerase chain reaction **TF**, transcriptional factor; **TNV**, *Tobacco necrosis virus*; **TRV**, *Tobacco rattle virus*; **TuMV**, *Turnip mosaic virus*

CONTENTS

DISEASE IN PLANTS

The meristematic cells of a healthy plant divide and differentiate as needed, while different types of specialized cells absorb water and nutrients from the soil; translocate these to all plant parts; carry on photosynthesis, move, metabolize or store photosynthetic products; and produce new reproductive structures for survival and multiplication. When a pathogenic organism interferes with the ability of cells or a plant part to carry out one or more of these essential functions, the activities of the cells are disrupted, altered, or inhibited and the plant becomes diseased. This disease, which is the outcome of a successful infection, rarely kills a plant, if the plant is not infected by a necrotroph pathogen. At first, the infection is localized in one or a few cells and is invisible. Soon, however, the reaction becomes more widespread and affects plant parts, developing changes that are visible to the naked eye. These visible changes are the symptoms of the disease. The visible or otherwise measurable adverse changes in a plant produced by the pathogen infection are a measure of the degree of disease in the plant (Gómez-Gómez 2004). Thus, the disease in plants can be defined as the series of invisible and visible responses of plant cells or tissues to the pathogen that result in adverse changes in the form, function, or integrity of the plant and may lead to the death of plant parts or the entire plant. The kinds of cells and tissues that become affected determine the type of physiological function that will be disrupted (Agrios 2005). For example, infection of roots may cause roots to rot and make them unable to absorb water and nutrients from the soil. Infection of xylem vessels, as happens in vascular wilts interferes with the translocation of water and minerals to the crown of the plant. Infection of leaves, as happens in leaf spots, blights, rusts, mildews, mosaics and so on, interferes with photosynthesis, while infection of phloem cells in the veins of leaves and in the bark of stems and shoots, as happens in crankers and in diseases caused by virus, interferes with the downward translocations of photosynthetic products. The infection of flowers interferes with the proper development of reproductive organs. In addition, each kind of pathogen has evolved a particular way to invade plants. Some species directly penetrate surface layers by using mechanical pressure or enzymatic attack. Others pass through natural openings, whereas a third group invades only wounded tissues.

Crocus sativus is cultivated for its red style branches,

Fig. 1 Representative samples of *C. sativus* corms severely affected by fungi.

which once dry constitute the saffron spice. *C. sativus* is a triploid sterile plant, propagated by corms. As a subterranean organ, the corm is susceptible to diseases caused by fungi, bacteria, nematodes and viruses (**Fig. 1**). Infected plants die off early, resulting in reduction of corm yield, quality and flower and stigma production. In the following sections we will cover the different pathogens that have been isolated and identified in *C. sativus* and also the strategies which the plant has developed to deal with them.

FUNGI AND OOMYCETES AS SAFFRON PATHOGENS

Fungi and oomycetes cover the majority of eukaryotic plant pathogens, but they represent less than 2% of the approximately 100,000 known fungi and oomycetes species. Both microorganisms show filamentous growth in their vegetative stage, produce mycelia and form spores by asexual and sexual reproduction. Almost all pathogenic fungi spent part of their lives on their host plants and part in the soil or in plant debris on the soil. Thus, these pathogens show different lifestyles and are classified as biotrophs, necrotrophs and hemitrophs. Biotrophs grow and reproduce in living plant tissue and obtain nutrients through intimate interactions with living plant cells. They are regarded as having an intricate biological interaction with their host plants, presumably as a result of co-evolution, since they exhibit a high degree of specialization for individual plant species. Necrotrophs, which feed on dead plant cells, kill host cells by means of toxic molecules and lytic enzymes, subsequently decomposing the plant tissue and consuming it for their own growth (van Kan 2006). The hemitrophs that initially establish a biotrophic relationship with their host, whose cells die as the infection proceeds (Latijnhouwers *et al*. 2003). This switch is usually triggered by increasing nutritional demands as the fungal biomass increases. Some of the world's most devastating phytopathogenic species fall into this category. Finally some fungi are facultative parasites because they can live, grow and multiply perfectly in the soil or elsewhere as saprophytes, although when the conditions are favourable, they have the ability to parasitize and cause disease in the plant. Most of the fungi that have been isolated from *C. sativus* plants belong to this group (**Table 1**).

Uromyces croci is a basidomycetes fungus, belonging to Uredinales order, mainly recorded as attacking only the leaves of *Crocus* species in the Mediterranean countries, developing the so-called crocus rust disease. However, leaf infections are not known to occur in northern Europe, where instead the corms are affected by Teleutospores present in soil. The association of the mycelium with the vascular tissue of the host can lead to the production of an infection in new corms (Boerema and Kesteren 1965).

The genus *Aspergillus* is a diverse and familiar group of ascomycetes which is mostly saprophytic, but includes human pathogens, plant pathogens, and species useful in industrial processes and genetic research. *Aspergillus niger* (**Fig. 2A**) is a fungi found worldwide which is responsible for black mould (**Fig. 2B**). It is transmitted by contaminated

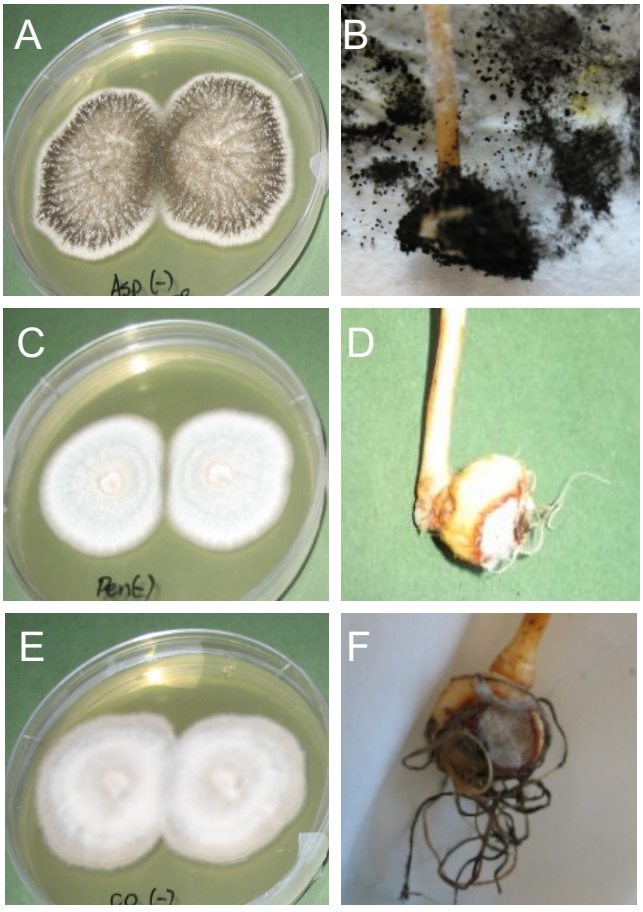

Fig. 2 Fungi as saffron pathogens. (**A**) *Aspergillus niger* isolated from infected saffron corms. (**B**) Saffron corms affected by *A. niger*. (**C**) *Penicillium sp* isolated form saffron corms. (**D**) Saffron corms affected by *Penicillium*. (**E**) *Cochliolobus sp* isolated from infected saffron corms. (**F**) Saffron corms affected by *Cochliolobus.*

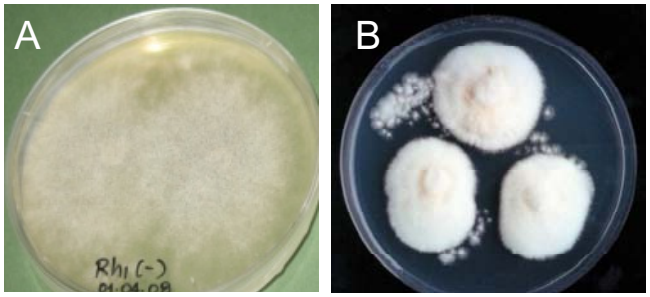

Fig. 3 Other fungi isolated from saffron corms in Spain. (**A**) *Rhizopus sp* isolated from infected saffron corms. (**B**) *Beauveria sp* isolated from infected saffron corms.

corms or soil, with the infection usually starting at the root initiation stage and continuing through corm storage.

Several *Penicillium* species have been isolated from infected saffron corms (**Fig. 2C, 2D**). *Penicillium* rot, more commonly referred to as blue mould rot, is one of the primary agents responsible for crop losses of flower bulbs and vegetables during storage. *Penicillium corymbiferum* Westl., *P. crocicota* Yamamoto and *P. chrysogenum* have been reported as pathogens in crocus (Moore 1939; Yamamoto *et al*. 1956; Saaltink 1971). Saffron plants infected with *P. corymbiferum* manifest damping-off, basal stem rot and drooping or wilting of shoots; the corms often have dark lesions beneath the outer tunic and, sometimes, a blue-green mould on their surface (Capelli *et al.* 1991). It has been demonstrated that a very heavy infection of *P. corymbiferum* on crocus corms at planting can reduce harvested corm yield by more than 20% (Sutton and Walle 1985). The

Table 1 Fungi and oomycetes isolated from *Crocus* in different geographic locations.

Fungi	Life style	Location	Reference
Aspergillus niger	facultative parasite	Spain	This work
Beauveria	facultative parasite	Spain	This work
Botrytis	necrotrophic	The Netherlands	Boerema and Hamers 1989
Burkholderia gladioli	hemibiotrophic	Argentina	Wit *et al*. 2002
Cladosporium	facultaive parasite	Spain	This work
Colchiobolus	hemibiotrophic	Spain	This work
Fusarium oxysporum f.sp gladioli	hemibiotrophic	The Netherlands	McClelland 1945
		Japan	Yamamoto 1954
		Germany	Mes *et al*. 1994
		Italy	Cappelli 1994
Fusarium oxysporum f.sp croci	hemibiotrophic	The Netherlands	Boerema and Hamers 1988, 1989
Fusarium oxysporum f.sp tuberosi	hemibiotrophic	Spain	Castillo and Gómez-Gómez 2009
Fusarium moniliforme	hemibiotrophic	India	Personal communication
		Spain	This work
Penicillium corymbiferum	facultative parasite	UK	Yamamoto *et al*. 1956
		Italy	Capelli *et al*. 1991
Penicillium crocicola	facultative parasite	Japan	Yamamoto *et al*. 1956
Penicillium cyclopium	facultative parasite	Italy	Cappelli and Di Minco 1999
Penicillium spp.	facultative parasite	Spain	This work
Phytium irregulare	necrotrophic	The Netherlands	Van Os *et al*. 1998
Phytium spp.	necrotrophic/ saprophytic/ facultative parasite	The Netherlands	Schenk 1969
Rhizoctonia violacea	necrotrophic and hemibiotrophic	Spain	Pérez-Bueno 1995; De Andrés 1998
Phoma spp.	necrotrophic	India	Madan *et al*. 1966
		The Netherlands	Boerema 1976
		Spain	Pérez-Bueno 1995
Rhizoctonia crocorum	necrotrophic and hemibiotrophic	Greece	Goliaris 1999
Rhizopus nigricans	facultative parasite	Spain	This work
Sclerotium rolfsii	necrotrophic and hemi-biotrophic	India	Kalha *et al*. 2007
Stromatinia gladioli	necrotrophic and hemibiotrophic	The Netherlands	Schenck 1970
Uromyces croci	biotrophic	The Netherlands	Boerema and Van Kesteren 1956

corms grown from infected parent material tend to be smaller than those produced from a clean stock, which would account for the reduced yields. Infection by this fungus has been reported in Italy (Cappelli *et al*. 1991), in Japan (Yamamoto *et al*. 1956; Gould and Miller 1971; Saaltink 1971), Scotland (Sutton and Wale 1985) and Spain (this work) (**Table 1**).

Rhizopus spp are zygomycetes, a primitive fungi, which are either saprophytes or weak parasites of plants and plants products where they cause soft rots or moulds. These fungi enter plants through wounds present on the surface. *Rhizopus nigricans* (**Fig. 3A**) has been isolated from wounds present in *C. sativus* corms (the work in this manuscript).

Beauveria is a well known facultative pathogen and an entomopathogenic fungus. Although the species isolated from *C. sativus* has not been determined (**Fig. 3B**), among the *Beauveria* species identified, *B. bassiana* has been environmentally approved for commercial use against a variety of agricultural pests, including whiteflies, beetles, grasshoppers and psyllids. *B. bassiana* has also been used as a model system to study fungal-mediated tick (Acari: Ixodidae) biological control. The *Beauveria* lifestyle is further unique in that it is a facultative saprophyte and can exist as a plant endophyte and/or form intimate interactions with plants (White *et al*. 2002).

Common root rot is caused by *Cochliobolus* (**Fig. 2E, 2F**) species and produce symptoms identical to the *Fusarium* organism. Infected plants are usually scattered throughout fields rather than in patches and often go unnoticed. Severely infected plants ripen prematurely and "stick out" in green stands. Root development is reduced and plants are easy to pull out of the soil. Lower stems, leaf sheaths, and roots have brown lesions, resulting in a reduced number of new corms.

The genus *Fusarium* comprises several fungal species widely distributed in soils and organic substrates. One of the most relevant species of this genus is the ascomycete *Fusarium oxysporum* (**Fig. 4A**), which causes vascular wilt and root rot in more than 100 species of plants (Berrocal-Lobo and Molina 2008). *Fusarium* corm rot incited by *F. oxysporum* is the most destructive disease in saffron, having caused severe yield losses in Italy (Cappelli 1994). The disease has been referred to by various names, including dry rot, brown rot, basal rot and yellows. The major symptoms of the disease occur during the flowering time when the infected plants show drooping, damping-off, yellowing and wilting of shoots, as well as basal stem rot and corm rot (**Fig. 4B-D**). The pathogen survives in infected corms and in the soil as mycelium, chlamydospores, macroconidia and microconidia (Brayford 1996). Plants may become infected in the field, when germinating spores or mycelia enter the roots directly or through wounds. Most probably, the pathogen may be introduced into new saffron-growing regions via contaminated corms (Cappelli and Di Minco 1999). The disease was detected for the first time in Japan (Yamamoto *et al*. 1954) and was later reported in India (Shah and Srivastava 1984), Spain (García-Jiménez and Alfaro-García 1987) and Italy (Cappelli 1994). From the pathogens isolated from saffron, *Fusarium* has been detected in many different saffron cultivation areas generating the highest losses in corm yield.

The fungi *Rhizoctonia* and *Sclerotium* are soil inhabitant basidiomycetes, which cause serious diseases in saffron. The first report on *C. sativus* plants infected by *Rhizoctonia* was done in 1728, by Duhamel (Orlob 1964; Ecklund 1971), when this author was investigating a disease affecting saffron fields in Gâtinais (France), he discovered that the cause of the disease was a parasitical fungus who named *Tuberoides*, which was later on renamed *Rhizoctonia* by De Candolle (1815). The events occurring during the infection process of *Rhizoctonia* include adhesion, penetration, colonization and host reaction. When *Rhizoctonia* hyphae contact with the external surface of a compatible host, there is a recognition phenomenon that results in profuse hyphal branching and formation of infection structures. The initial steps of the infection process are characterized by both the adhesion of hyphae and an altered growth pattern resulting in directed hyphal growth and the formation of penetrating hyphal structures. Infection structures that are subsequently formed allow the fungus to reach intact plant tissue. In the

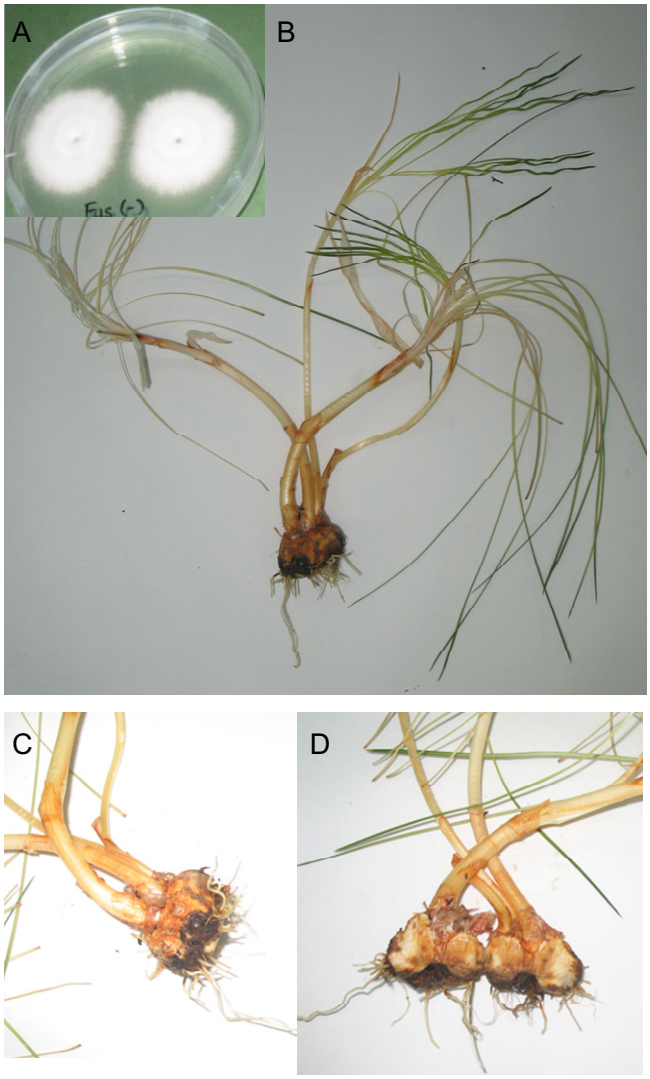

Fig. 4 *Fusarium oxysporum* as an important saffron pathogen. (**A**) *Fusarium oxysporum* isolated from disease saffron corms. (**B**) Symptoms developed by saffron plants caused by *F. oxysporum*. (**C**) Corm-rot and root-rot caused by *F oxysporum* in infected plants. (**D**) Longitudinal section of affected corms.

course of infection, substances are exchanged between the pathogen and the host plant. These include materials such as extracellular fungal enzymes and host exudates (reviewed by González-García *et al*. 2006). After penetration, colonization of plant tissue is accomplished by the production of hydrolytic enzymes capable of degrading several cell walls beyond the advancing hyphae. Together with cell wall damage, changes in the cytoplasm of cortical cells can be detected before colonization events are produced. From a cytological point of view, pathogenesis in *Rhizoctonia* is characterized by severe damaging or killing of plant cells, before or immediately after penetration and colonization. Thus, as in other fungal pathogen models, penetration and colonization is regarded as a primary process of hyphal growth into highly degraded plant tissue, suggesting a combination of both necrotrophic and hemi-biotrophic behaviour for this fungus on its compatible hosts. In saffron corms, *Rhizoctonia* causes rotten brown areas that may be superficial or may extend inward to the middle of the root system, with severely infected plants having practically no root system. The rotting tissues usually decompose and dry, forming a sunken area filled with the dried plant parts mixed with the fungus mycelium and sclerotia.

The presence of *Sclerotium* as a pathogen in saffron has only been recently reported in India (Kalha *et al*. 2007). In newly infested fields, the disease occurred in small patches that gradually enlarged each year. Symptoms appeared as brown-to-dark brown sunken, irregular patches below corm scales. Lesions were usually 1 mm deep with raised margins. Severely infected corms had foliage that dried from the tip downward, and white fungal mycelia appeared on the bulbs that rotted at later stages of disease development.

The oomycete *Phytium* is not a true fungus but is a fungal-like organism. Several species are facultative parasites of plants and of animals. Some species are parasites of other fungi, and others are primarily saprophytes (Tambong *et al*. 2006). *Pythium* species are ubiquitous non-specific soil-borne vascular pathogens, which under wet, humid conditions cause diseases in many plants including agronomic crops. They possess a high level of diversity with more than 200 species described (Souza *et al*. 2003). Several species of *Pythium* cause severe diseases as sole pathogens or in complexes with *Fusarium* spp. and *Rhizoctonia* spp. Many *Pythium* species can occur simultaneously at the same site and, often, more than one species can often infect a certain host plant. Infections occur on roots, lower stems and soft plant tissues, which are naturally present in seedlings and germinating bulbs and corms, causing root rot (Schenk 1970), stem rot and seedling damping off. Tissue degradation is caused by both cell wall degrading enzymes such as pectinases, hemicellulases, cellulases and proteinases and toxins produced by *Pythium* (Martin 1994). In *Crocus* plants affected by *Pythium irregulare* initially infected roots showed a distinct brown discoloration at the tip. Interestingly, *Crocus* roots are susceptible to the pathogen only during a short period after planting. Therefore, an epidemic may occur when periods of host susceptibility, pathogen activity, and suitable biotic and abiotic environments coincide (Van Os *et al*. 1998).

Stromatinia gladioli (Drayt.) Whetz. is a root-infecting fungus attacking several genera of the *Iridaceae*, notably *Gladiolus*, *Crocus* and *Freesia* (Gould 1958). In the past years the incidence of the disease in commercial crops has declined, following the introduction of routine corm treatments using benzimidazole fungicides (Humphreys-Jones 1971). However, this pathogen is highly persistent in field soils by means of sclerotia, which can remain dormant for long periods of time (5-10 years) and can act as a source of infection for future host plants. Excessive moisture or abundant rainfall and cooler temperatures increase the severity of the disease.

Phoma species are common soil-inhabiting fungi, which appear as wound- and weak parasites. They have been isolated in *Crocus* spp. (Boerema 1976) and *C. sativus* (Madan *et al*. 1966; Perez-Bueno 1955; and the work in this study).

Fungi of the genus *Botrytis* Persoon are important pathogens for many agronomically important crops, such as grapevine, tomato, bulb flowers, and ornamental crops (Jarvis 1977). *Botrytis* diseases appear primarily as blossom blights and fruit rots but also as leaf spots and bulb rots in the field and in stored products. *Botrytis* species are necrotrophs, which induce host-cell death resulting in progressive decay of the infected plant tissue. The pathogen produces abundantly sporulating gray mycelium on infected tissue. Macroconidia (mitotically produced spores) can be transported by wind over long distances. *Botrytis* overwinters in the soil as mycelium in decaying plant debris and as sclerotia, melanized mycelial survival structures. Some species frequently produce a sexual teleomorphic stage in which ascospores are produced in an apothecium. When collected in nature, apothecia are found under cool weather conditions, arising from sclerotia, which have developed on decayed plant parts in moist soil. *Crocus* blight is usually ascribed to *Botrytis croci* (Hennebert 1973, Boerema and Hamers 1989). In addition, it has been recorded that *B. gladiolorum* has been described as grey mould in *Crocus*. The conidial size of the fungus is very similar to that of *B. croci* occurring in crocus, but is distinguished by the production of sclerotia (Boerema and Hamers 1989).

VIRUSES

Plant cells have a robust cell wall which viruses cannot penetrate them unaided. Most plant viruses are therefore transmitted by a vector organism that feeds on the plant or (in some diseases) are introduced through wounds made, for example, during farming operations (e.g. tunic removing and corm progeny separation in the case of saffron). The largest and most significant vector group of plant viruses are insects, especially aphids, which transmit viruses from many different genera, including the *Potyvirus* and *Cucumovirus* viruses that infect *Crocus*. Symptoms of viral infection include tissue yellowing (chlorosis) or browning (necrosis), mosaic patterns, and plant stunting. Plant viruses are biotrophs that all face the same three basic challenges: how to replicate in the cell initially infected; how to move into adjacent cells and the vascular system; and how to suppress host defences and thereby colonize the entire plant.

Turnip mosaic virus (TuMV) has an RNA genome and infects a wide range of plant species, including those of the genus *Crocus* (Hu *et al*. 1996; Chen and Chen 2000). It is probably the most widespread and important virus infecting both crop and ornamental species, and occurs in many parts of the world including the temperate and tropical regions of Africa, Asia, Europe, Oceania and North/South America (Provvidenti 1996). TuMV belongs to the genus *Potyvirus*. This is the largest genus in the largest family of plant viruses, the *Potyviridae* (Ward *et al*. 1995), which in turn belongs to the picorna-like supergroup of viruses in animals and plants. TuMV, like other potyviruses, is transmitted by aphids in a non-persistent manner (Shukla *et al*. 1994). All potyviruses have flexuous filamentous particles 700–750 nm long, each containing a single copy of the genome, which is a single-stranded positive sense RNA molecule about 10000 nt in length. The genomes of potyviruses have a single open reading frame that is translated into a single large polyprotein, which is hydrolysed, after translation, into several proteins by virus-encoded proteinases (Riechmann *et al*. 1992). This virus has been detected in saffron plants from China (Chen and Chen 2000), New Zealand (Ochoa-Corona 2007), France (D'Agostino *et al*. 2007) and Spain (this study) (**Fig. 5**). In these two last studies the virus was detected during the sequencing of ESTs libraries from stigma tissue. The symptoms observed on *C. sativus*, with TuMV varied from chlorosis, mild to severe mosaic, and necrosis.

Another virus infecting *Crocus* ssp. from the same family as TuMV is the *Iris severe mosaic virus* (ISMV) (Langeveld *et al*. 1991; van der Vlugt 1994). This virus causes conspicuous chlorotic stripes and/or mosaic patterns in the leaves, and breaking in the flowers. Symptoms are usually more severe in plants grown at temperatures of 16°C or less, or in those also containing other viruses.

The *Narcissus mosaic virus* (NMV) is also a potyvirus recently found in *Crocus* ssp. by ELISA and RT-PCR (Miglino *et al*. 2005, 2007), although this virus was observed to have a very narrow host range. The symptoms observed are typical of virus infection: stunting, yellowing, necrosis, and flower colour breaking. However, the methodology employed enabled the correct identification of the virus agent. Using the ELISA and RT-PCR methodologies these authors also identified the presence of *Bean yellow mosaic virus* (BYMV) and ISMV. Serological results indicated that BYMV and ISMV were the most commonly encountered viruses, whereas *Tobacco necrosis virus* (TNV) and *Tobacco rattle virus* (TRV) were found occasionally (Miglino *et al*. 2005).

Cucumber mosaic virus (CMV), the type species of the genus Cucumovirus in the Bromoviridae family (Peden and Symons 1973), has been found in most countries of the world and has been identified as the causal agent of several disease epidemics. Its host range exceeds 800 plant species, making CMV one of the most important viruses due to its economic impact. The genome of CMV consists of three capped plus-sense single stranded RNAs (Rybicki 1995). CMV isolates have been classified into two subgroups based on visual symptoms, serology and nucleic acid hybridization tests, with the isolates from *Crocus* belonging to subgroup II (Chen *et al*. 2001). Symptoms consist in retarded growth, various mosaics, streaking, spotting, and distortion of flowers and leaves.

Other virus particles have been isolated and described in *Crocus*, but have not been identified (Ehrig *et al*. 1997). Sometimes the virus is restricted to certain parts of the plant (e.g. the vascular system; discrete spots on the leaf) without spreading throughout the plant to cause a systemic infection. Furthermore, infection does not always result in visible symptoms (such as *Carnation latent virus* and *Lily symptomless virus*, both members of the genus Carlavirus). The presence of additional viruses in *Crocus* could therefore be overlooked.

Fig. 5 *Crocus sativus* plants showing the symptoms caused by TuMV.

TRANSPOSABLE ELEMENTS AS GENOME PATHOGENS IN SAFFRON

Besides viruses, transposons can be considered as another group of special pathogens affecting the plant genome. The difference between transposons and viruses is that transposons simply hop around within the genome of a host cell, whereas viruses colonise new cells. Viruses, in contrast to transposons, can survive outside cells and invade new cells. To achieve this, they need some extra machinery compared to transposons. Active or inactive transposons constitute a large proportion of the repetitive DNA fraction of plant genomes which are dispersed throughout the genome. Transposable elements are separated into two major groups depending on their mode of transposition (Flavell *et al*. 1994). Class I transposable elements include retrotransposons and other retroelements, which are almost certainly viral in origin. These elements, like retroviruses, propagate intracellularly through transcription and translation. This Class I includes long terminal repeat (LTRs) retrotransposons: *gypsy*-like and *copia*-like retrotransposons (Xiong and Eickbush 1990), and non-LTR retrotransposons, which can be divided into LINEs (long interspersed nuclear elements) and SINES (short interspersed nuclear elements) (Schmidt 1999). Class II elements move as DNA copies and include the *Ac/Ds* (Activator/Dissociation), *En/Spm* (Enhancer/Suppressor) and *Mu* (Mutator) transposons, together with MITES, which are most likely non-autonomous transposons (Wessler *et al*. 1995).

The analysis of sequences from expression libraries obtained from corms and stigmas of *C. sativus* has allowed the identification of transposons and retrotransposons in its

Table 2 Transposable elements identified in *Crocus sativus*.

Transposable elements	GeneBank accession number	Library
Polyprotein	Ex148312, Ex148595	stigma
Transposon	Ex148329, Ex148296	stigma
Transposon	BM005533, BM005531, BM005532	corm
Retrotransposon	BM005537, BM956290	corm
Ty-copia	BM005527, BM005538, BM005528, BM005534, BM005530, BM956292	corm
Ty3-gypsy	BM005529, BM005535, BM005526, BM005536, BM956293, BM956291	corm

genome, from Class I and Class II (**Table 2**). Transpositions are influenced by developmental and perhaps environmental signals and may play a role in temporal and spatial patterns of gene expression.

NEMATODES

More than 20 genera of nematodes, i.e. round worms approximately 1 mm in length, cause plant disease. All the sedentary endoparasitic nematodes have evolved the ability to induce morphological changes in plant cells to form feeding cells (Sijmons *et al*. 1994). The juvenile form migrates through the soil towards the root system. Once it reaches the root epidermis, it enters the plant preferentially in the differentiation and elongation zone by perforating cells using a combination of intensive stylet thrusting, enzymatic softening of the cell walls and mechanical force. The nematode then begins migration towards a site in the plant for suitable feeding. At this time, these nematodes become immobile and completely dependent on the successful induction and maintenance of specialized feeding cells. This intimate relationship persists for approximately two months until the end of the nematode's life cycle. These parasites are therefore biotrophic: they do not kill the cells they feed from but instead modify them into efficient food sources. The mechanism of feeding cell formation is different and specific for each infecting nematode.

The nematode *Dytilenchus destructor* severely affects bulbs, corms and tubers (Ortuño and Oros 2002). *D. destructor* is unable to withstand excessive desiccation, and for this reason is usually only important in cool, moist soils. Because nematodes attack only subterranean and not aerial parts of plants, there are generally no obvious symptoms in the aerial parts of the plant. In the tissues affected, feeding initially produces yellow necrosis lesions, which then turn dark-brown. These lesions can be observed in the corms when cut longitudinally or transversally. In advance stages of disease, the corms are also infected by other parasites, especially fungi, which infect the plant and finally mask the responsibility of the nematode for the observed fall in yield (Melakeberhan 2003).

Pratylenchus penetrans, known as lesion nematode or meadow nematode, and *P. pratensis* have been identified in *Crocus* spp. and *C. sativus* (Metkalf 1903; Schenk 1970). These nematodes attack the external cells of radicles and penetrate the tissues little by little whatever the stage of development. They create cavities in which they reproduce and tissues may contain thousands of individuals. The parasites gradually make their way to parts of the parenchyma that are still sound, with the affected areas being rapidly destroyed by a characteristic necrosis. When conditions are unfavourable, e.g. the roots decompose; nematodes leave the root and travel freely within the soil until they come across another host root.

The nematode *Aphelenchoides subtenuis* was identified for the first time by Cobb in 1926 (Steiner and Buhrer 1932) in *Narcissus* bulbs, but was classified as a bud and leaf nematode, although studies on bulbous plants showed that *A. subtenuis* behaves in these plants as a root nematode. *A. subtenuis* has been detected in corms and leaves of *Crocus* spp. in different countries (Koliopanos and Kalyviotis-Gazelas 1979; Decker 1989; Ortuño and Oros 2002; McCuiston *et al*. 2007). It is not strictly an endoparasite because it can survive in dried leaves, but not in the soil.

Recently, a PCR-based approach has detected *Meloidogyne chitwoodi* and *M. fallax* which affect root growth and yield in *Crocus* plants (Zijlstra and Van Hoof 2006), even though these plants are poor hosts. Both nematode species spend the winter as eggs or juveniles and can survive extended periods of sub-freezing temperatures.

PLANT DEFENCE RESPONSES

The surfaces of plant organs, both above and below ground are continuously and permanently exposed to a diverse range of enemies, including microbial pathogens, nematodes and insects. As a result, plants have evolved intricate mechanisms to recognize and defend themselves against the wide array of these disease-causing agents. Plants can directly detect the pathogen presence of elicitors, either by non-self recognition of PAMPs, which are molecules that are highly characteristic of a whole class of microorganisms (Gómez-Gómez and Boller 2002); or by monitoring the integrity of their own cell walls (i.e. 'intact self' or 'degraded self') (Hèmaty *et al*. 2009). This recognition takes place through receptor molecules present in the plant, which are specific to the elicitor molecule (Gómez-Gómez 2004).

After pathogen perception, two types of plant resistance response to potential pathogens can be distinguished: the non-host resistance response (frequent); and the race/cultivar-specific host resistance response (comparatively rare). In the non-host resistance response, plants are equipped with a variety of defence mechanisms, including preformed defences such as waxes, cell wall components, and secondary metabolites. Upon pathogen detection, plants activate a number of responses that can include a hypersensitive response (HR; rapid localized cell death at the site of infection), increased expression of defence related genes [e.g. pathogenesis-related (PR) genes], and the oxidative burst (Gómez-Gómez 2004).

PREFORMED OR PASSIVE DEFENCE MECHANISMS IN SAFFRON

Preformed defence is the first obstacle a pathogen faces when invading a plant. For example, the plant cell wall, and in the case of saffron the presence of the fibrous tunic, protects the corm from pathogens, insects and water loss. But plants constitutively produce a plethora of secondary metabolites, many of which can act as antimicrobial compounds during defence against microorganisms (Dixon 2001). These compounds may be present in their biologically active forms or may be stored as inactive precursors that are converted to their active forms by host enzymes in response to pathogen attack or tissue damage. These compounds include saponins, phenolics, cyclic hydroxamic acids, cyanogenic glycosides, isoflavonoids, sesquiterpenes, sulphur-containing indole derivatives and many others. Altogether, these compounds represent the first chemical barriers to infection and are associated with non-host resistance. Saponins are glycosylated triterpenoid, steroid, or steroidal alkaloid molecules with antifungal activity (Osbourn 1996). Saponins are constitutively produced in many plants and can also be induced as a result of a pathogen infection. Saffron is characterized by the presence of saponins in stigma (Hosseinzadeh and Younesi 2002) and in corm tissue (Rubio-Moraga 2003) where they can play antifungal roles. Saffron corms also show the presence of 12

Table 3 Defence genes identified in *Crocus sativus*.

Protein	GenBank accession numbers	Mechanism of action	Library
PR-1	EX146574	Unknown.	stigma
PR-2	EX148616, EX146887	Hydrolysis of the structural 1,3-β-glucan present in the fungal cell wall.	stigma
Acidic chitinase	BM005616	Cleave cell wall chitin polymers *in situ*.	corm
Chitinase	EU446024	Cleave cell wall chitin polymers *in situ*.	corm
Chitinase	BM005637	Cleave cell wall chitin polymers *in situ*.	corm
Chitinase	EX146541	Cleave cell wall chitin polymers *in situ*.	stigma
PR-5	EX147388	Not completely understood. Some cause fungal cell permeability changes, others bind to 1,3-β-glucan and exhibit 1,3-β-glucanase activity.	stigma
PR-5	BM005643	Not completely understood. Some cause fungal cell permeability changes, others bind to 1,3-β-glucan and exhibit 1,3-β-glucanase activity.	corm
PR-10	Ex147729, Ex148310, EX147960	Ribonuclease activity.	stigma
PR-12	EX146849	Fungal inhibition probably occurs through an ion efflux mechanism.	stigma
PR-13	BM005615	Fungal inhibition probably occurs through an ion efflux mechanism.	corm
LTP (PR-14)	BM956322	Lipid transfer protein. Probably involved in plant-fungi interactions.	corm
LTP (PR-14)	EX148692, EX148514, EX148522, EX148506, EX148483, EX148428, EX148387, EX148382, EX148282, FJ997554, FJ997555	Lipid transfer protein. Probably involved in plant-fungi interactions.	stigma
WRKY2	EX148608	Activates the transcription of PR encoding genes.	stigma
WRKY4	EX144622	Activates the transcription of PR encoding genes.	stigma
Polygalacturonase inhibitor	BM005638, BM005626	Inhibition of fungal polygaraturonases.	corm
Elicitor response	BM005628		corm
PAL	EX147644.1	Phenylalanine ammonia-lyase.	stigma
Peroxidase	EX148603		stigma
β-1,3-galactosyltransferase	EX148441, EX146940, EX147392	Elicitor response protein.	stigma
SOD	EX147952	Superoxide dismutase.	stigma

PR: pathogenesys related; LTP, lipid transfer protein; PAL, phenylalanine ammonia-lyase

different phenolic compounds of which pyrogallic acid, kaempferol, p-coumaric acid and gallic acid, involved in stress response, have been tentatively identified (Crungoo *et al*. 1986; Ebrahimzadeh *et al*. 1997). Furthermore, *C. sativus* phenolic extracts show important antimicrobial activity (Senghum *et al*. 2009) and phenolic compounds together with peroxidases and catalases counteract reactive oxygen species (ROS) in order to survive and prevent molecular harm and damage by microorganisms, insects, and herbivores (Nicholson and Hammerschmidt 1992). Peroxidase, catalase and superoxide dismutase activities have been detected in saffron corms in different developmental stages (Keyhani and Keyhani 2004; Keyhani *et al*. 2006), and genomic approaches have enabled the identification of partial sequence homologues for these enzymes (**Table 3**).

INDUCIBLE DEFENCE MECHANISMS IN SAFFRON

The second obstacle an invading pathogen has to face is the inducible plant defence mechanisms. The induced mechanisms are associated with local changes at the site of pathogen infection, such as the hypersensitive response (HR), one of the most efficient forms of plant defences (Dixon and Paiva 1995). Besides causing accumulation of antimicrobial compounds, such as phenolic compounds, phytoalexins (Ortega *et al*. 2005) and antimicrobial peptides, HR also leads to an increase in the activity of peroxidases (Kortekamp and Zyprian 2003) and polyphenol oxidase enzymes (Agrios 2005) involved in defence responses (Thipyapong *et al*. 2004). The response to a pathogen also involves transcriptional activation of numerous defence-related genes, opening of ion channels, modifications of protein phosphorylation status, and activation of preformed enzymes to undertake specific modifications to primary and secondary metabolism (**Fig. 6**). In addition, a range of secondary signalling molecules are generated to ensure coordination of the defence response both temporally and spatially, resulting in rapid containment of the pathogen.

As previously stated, phenolic compounds, namely pterocarpans, coumarins, flavonols, and isoflavones (Dixon and Paiva 1995; Harbone 1999), are an important group of secondary metabolites involved in resistance to pathogens due to their antimicrobial activity, which biosynthesis increases after pathogen detection. The accumulation of phenolic compounds at the challenge site is a common feature of cell wall reinforcement in plant-microbe interactions. Cell wall reinforcements are generally accompanied by localized production of reactive oxygen species (ROS), which drive cell wall cross-linking, have antimicrobial activity, and are involved in defence-related signalling (Bradley *et al*. 1992; Levine *et al*. 1994). Besides, ROS responses orchestrate the HR response. The HR is one of the earliest visible manifestations of induced defence reactions and resembles programmed cell death in animals (Dangl *et al*. 1996). Concurrent with HR development, defence reactions are triggered in both local and distant parts of the plant and accompanied by a local and systemic increase in endogenous salicylic acid (SA) levels and the upregulation of a large set of defence genes, including those encoding pathogenesis-related (PR) proteins. The PR proteins are not or only present at basal concentrations detectable in healthy tissues, but upon pathological conditions, accumulation at the protein level is detected. These proteins encompass several different families of structurally and functionally unrelated proteins. There are 17 PR families: the PR-1 and PR17 proteins, whose biological function is still enigmatic; β-1,3-glucanases (PR-2), plant chitinases which are represented by several families (PR-4, PR-4, PR-8, PR-11), thaumatin-like proteins (PR-5), proteinase-inhibitors (PR-6), endoproteinases (PR-7), peroxidases (PR-9), ribonuclease-like proteins (PR-10), defensins (PR-12), thionins (PR-13), lipid transfer proteins (PR-14), oxalate oxidases (PR-15) and oxalate oxidase-like (PR-16). Although some of these PR proteins exhibit potential *in vitro* antimicrobial activities and their accumulation in plants is related to plant resistance responses, a direct functional role in defence could not be demonstrated for all (Sels *et al*. 2008). The target structures of the antifungal proteins range from the outermost part of the fungal cell, the cell wall, to the plasma membrane and finally to several intracellular targets. Therefore, these proteins exhibit a very wide diversity of action mechanisms, including, for example, inhibition of the synthesis of the fungal cell wall or disruption of its structure and/or function, membrane channel and pore formation, damage to cellular ribosomes, inhibition of DNA synthesis and inhibition of the cell cycle (Theis and Stahl 2004).

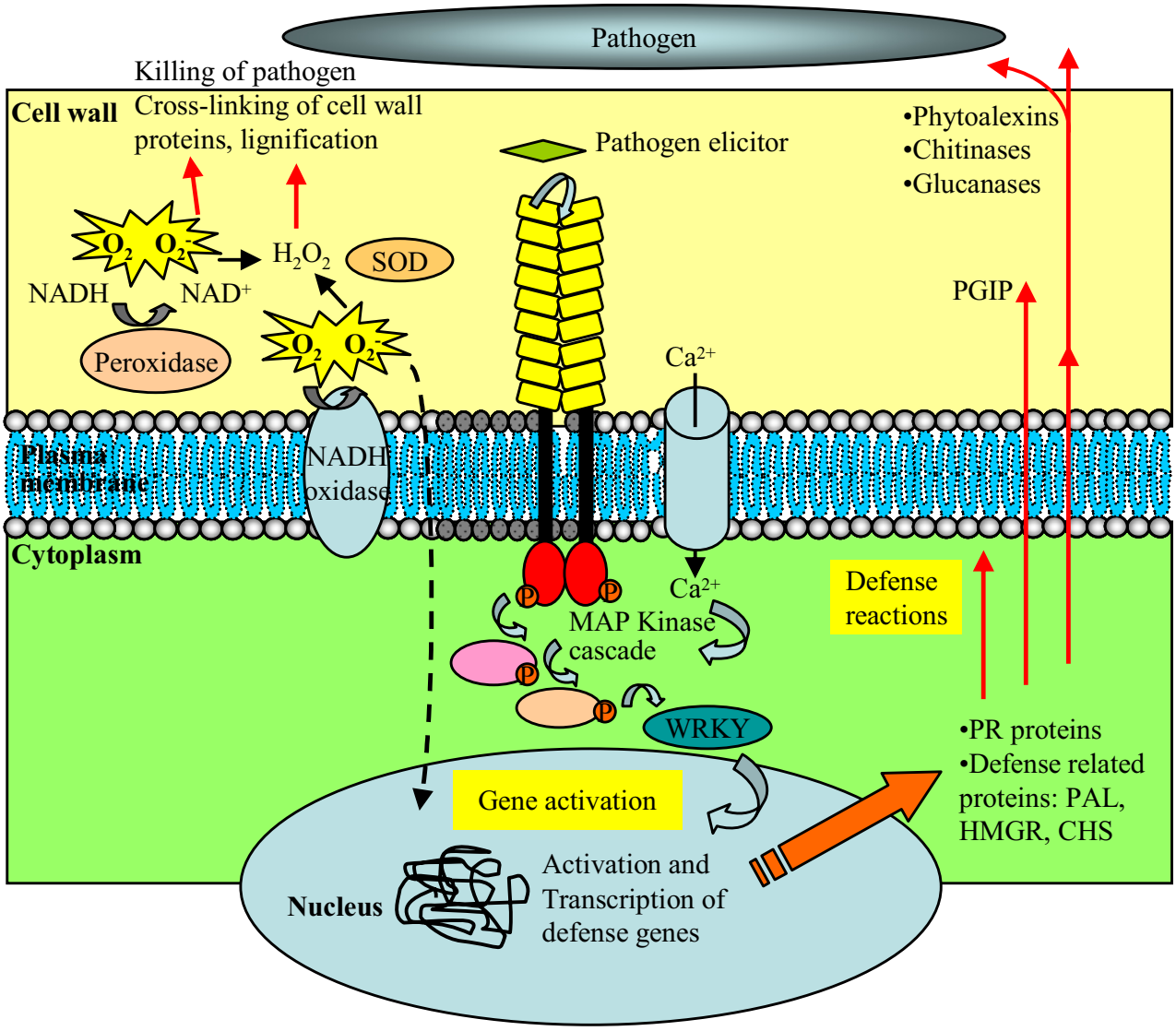

Fig. 6 Overview of the plant cell signalling components mediating responses upon pathogen attack. Plant cells possess a variety of membranes receptors that perceive endogenous (e.g. plant cell wall (CW)-derived fragments) or exogenous (e.g. PAMPs) signals, which can be either peptides or oligosaccharides (or both in the case of proteoglycans). A given ligand activates a specific receptor, which initiates downstream signalling events (a MAP kinase cascade). Most pathogens trigger a common/ interconnected plant signalling network. The graded transcriptional responses associated with the defence response clearly indicate the existence of a complex regulatory circuitry comprising transcriptional activators and repressors fine tuning the expression of defence genes. CHS, chalcone synthase; HMGR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; PAL, phenylalanine ammonia-lyase; PGIP, Polygalacturonase inhibiting protein; SOD, superoxide dismutase.

Nevertheless, the mode of action of most of these proteins remains to be elucidated *in vivo*.

The analysis of corm and stigma libraries from saffron have identified several protein-encoding genes associated with defence responses (Álvarez 2003; D'Agostino *et al*. 2007) (**Table 3**). Antimicrobial proteins, PR, involved in virus resistance and in fungi recognition identified in corm and stigma, could be implicated in the resistance response of *C. sativus* to pathogen infection. Among the PR protein families, the different classes of chitinase conform an heterogeneous group. In fact, a new chitinase from corms, which shows antifungal activity, has recently been isolated (Castillo and Gómez-Gómez 2009). Chitin is the major cell wall component in filamentous fungi (BeMiller 1965). It is therefore not surprising that *C. sativus* synthesizes a large number of defence proteins capable of binding to chitin and chitin oligosaccharides (**Table 3**).

The expression of *PR* and defence related genes are modulated by members of several transcription factor (TF) families. In particular, zinc-finger type WRKY factors play a broad and pivotal role in regulating defences (Eulgem and Somssich 2007). Members of this family contain at least one conserved DNA-binding region, designated the WRKY domain, comprising the highly conserved WRKYGQK peptide sequence and a zinc finger motif (CX4–7CX22–23HXH/C). This domain generally binds to the DNA element termed the W box (C/TTGACT/C).The majority of the analysed WRKY genes respond as well to pathogen attack (Pamdey and Somssich 2009). Two homologues to WRKY TF have been identified in *C. sativus*: WRKY2 and WRKY4 (**Table 3**). Ectopic expression of grapevine VvWRKY2, the homolog to saffron WRKY2, resulted in enhanced resistance to the necrotrophic fungi *Alternaria tenuis*, *B. cinerea*, and *Pythium* (Mzid *et al*. 2007), while the homolog to WRKY4 in *Arabidopsis thaliana* also plays a positive role in plant resistance toward necrotrophic pathogens (Lai *et al*. 2008).

CONCLUSIONS

Many different classes of pathogens including viruses, nematodes and especially fungi severely affect saffron yield and quality. While new pathogenic fungi, e.g. *Fusarium*, are currently being described as important saffron pathogens, there are no recent reports on detection associated to important losses caused by historic fungi, such as *Rhizoctonia*. Surprisingly, pathogenic bacteria have not been detected as responsible for important saffron losses, suggesting the

presence of important defence barriers in saffron, which prevent bacteria colonization. Genomic approaches have permitted the identification of several defence genes in saffron, although a number of important previously known genes are still missing, as is the case of elicitor-receptors for pathogen perception and recognition. Future research on saffron pathogenesis will aim at identifying more of these genes as well as their pathogen-derived elicitors, thus enabling the discovery of their plant receptors.

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How to reference: Author name(s) (2010) Title of chapter. In: Husaini AM (Ed) Saffron. Global Science Books, UK, pp. X-XX

Anticancer, Antimutagenic and Antioxidant Potential of Saffron: An Overview of Current Awareness and Future Perspectives

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ABSTRACT

Spices are dietary constituents consumed daily by most of the world population to enhance the flavor or taste of food. Saffron, obtained from dried stigmas of *Crocus sativus* L., is a commonly used spice for flavoring and coloring foods in different parts of the world. Since time immemorial, it has also been used in traditional medicine for various ailments. The chemical composition of saffron shows that it is a rich source of carotenoids. The three main chemical components of saffron are the bright yellow coloring carotenoids, crocin, a bitter taste, picrocrocin, and a spicy aroma, safranal. Crocetin and its semi-natural derivative, dimethylcrocetin (DMC), are also important carotenoids of saffron. During the past few years the anti-tumoural properties of saffron extracts, both *in vitro* and *in vivo*, have been demonstrated. *In vitro* investigations have reported saffron-mediated selective inhibition of the growth of tumor cells without damaging normal cells. A number of studies have demonstrated the saffron and its constituents exert a significant inhibition in the synthesis of DNA and proteins, and disruption of DNA–protein interaction on different malignant cells. Findings from various laboratories including ours have shown that saffron extract and some of its constituents possess antioxidant properties and can inhibit the genotoxicity or carcinogenicity of chemicals with various mechanisms of action. In view of the above findings and wide spread use of saffron, further research is needed to identify the active constituent(s) of saffron and elucidate the mechanism of action. This review provides an overall view on the biological properties of saffron with special emphasis on its anticancer, antimutagenic and antioxidant potential thus providing current awareness on saffron in biology and medicine and possible future perspective.

Keywords: anti-genotoxicity, carotenoids, chemoprevention, crocetin, crocin, *Crocus sativus*, saffranal

CONTENTS

INTRODUCTION

Saffron, the dried and dark red stigmas obtained from the flowers of *C. sativus* L., is an ancient, mystical spice that has been in use since the Greek-Minoan civilization. It has been historically used as a food colourant, drug in medicine and in cosmetics (Mathew 1982; Basker and Negbi 1983; Bowles 1985; Behnia *et al*. 1999). Numerous sources indicate that saffron cultivation is very old dating back to 2500–1500 BC, and originated possibly in Iran, Asia Minor or Greece and later became widespread in India, China, the Mediterranean basin and Eastern Europe (Tammaro 1987; Negbi 1999; Grilli *et al.* 2004). It is now largely cultivated in France, Greece, India, Iran, Italy, Spain, China, Israel, Morocco, Turkey, Egypt and Mexico for its flavoring and medicinal perspectives.

C. sativus is a perennial, stem less herb of the large *Iridaceae* family. It is a sterile triploid (Karasawa 1933; Ghaffari 1986; Rìos *et al.* 1996), probably derived from the wild species *Crocus cartwrightianus* (Mathew 1982; Grilli *et al.* 2004) and grows best often in friable, loose, well-watered and well-drained clay calcareous soils. The plant is characterized by its narrow leaves and a fleshy bulb called corm which is about 3 cm in diameter and approximately 8 g in weight. These corms play a vital role in the propagation of the plant as it fails to produce seeds upon selfing or crossing, due to its triploidy. Saffron flower, the source of spice, is purple colored with three thread-like reddish

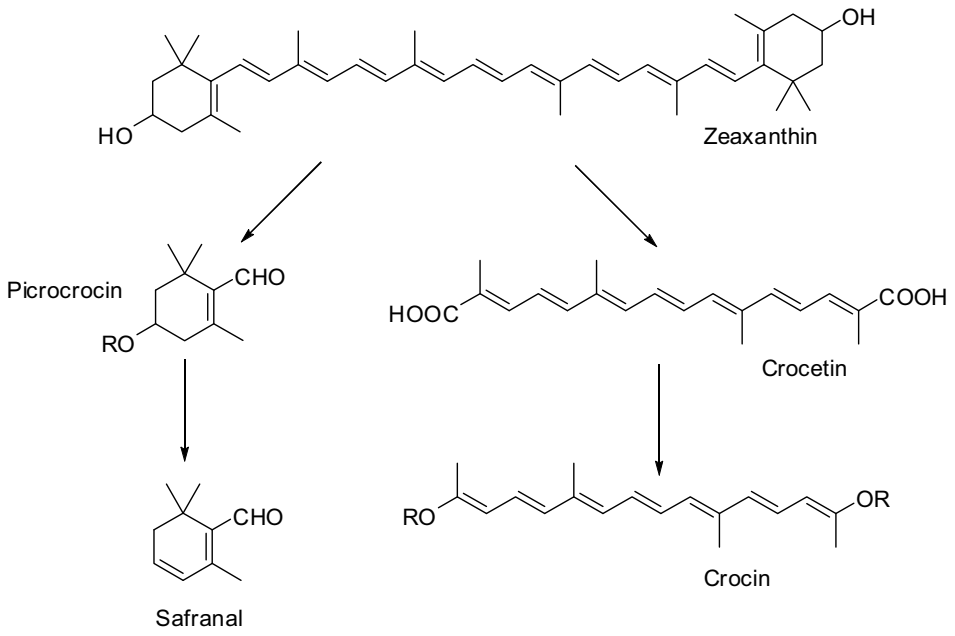

Fig. 1 Formation of saffron bioactive components from zeaxanthin bioactive cleavage.

coloured stigmas. These are collected, dried and processed to yield the most expensive spice (Rakesh *et al.* 2009).

Significant research has been conducted on the plant with the core interest of ascertaining its biologically active components and the possible mechanisms of their activity. These studies have concluded that saffron is the richest source of carotenoids and contains several volatile and non-volatile components derived from oxidative cleavage of carotenoids, of which the most important are: crocin, picrocrocin and safranal (Tarantilis *et al.* 1995; Escribano *et al.* 1996; Lozano *et al.* 2000). In addition, saffron contains proteins, sugars, vitamins, flavonoids and certain amino acids. It has now been recognized that the main constituents of saffron possess several pharmacological properties that can inhibit carcinogenesis (Nair *et al.* 1992; Escribano *et al.* 1996; Abdullaev and Espinosa-Aguirre 2004) and other major disorders in humans.

This review intends to provide an overall view on the biological activities of saffron to create awareness on its anticancer, antimutagenic and antioxidant potentials that can be explored and used as conventional and replacement therapies for treating various disorders in future.

ETHNO-MEDICAL IMPORTANCE

Saffron has not only been a primary spice but also a highly valued medicinal plant widely used in folk medicine. The therapeutic potential of saffron ranges widely, from treatment of simple ailments to treatment and prevention of obscure disorders. Traditionally it has been used as an antispasmodic, eupeptic, pain killer, anticatarrhal, carminative, diaphoretic, expectorant, stimulant, stomachic, aphrodisiac, and emmenagogue (Basker and Negbi 1983; Rios *et al.* 1996) and has shown profound effects against cramps, pain, asthma, bronchospasms, menstruation disorders, liver diseases, eye diseases and hypoxia. Evident reports show that saffron had been employed since ancient times to treat infertility and impotence (Abdullaev 2002; Chatterjee *et al.* 2005). Modern pharmacological studies reveals that saffron and its active components possess anti inflammatory, antitumor (Abdullaev 1993; Escribano *et al.* 1999a), antimutagenic, antioxidant, anti convulsant (Hosseinzadeh and Younesi 2002) and anti depressant potential (Hosseinzadeh *et al.* 2004). The crocin constituents has been reported to exert significant anti proliferation effects on certain human cancers (Aung *et al.* 2007). It has also been reported that crocin and safranal exhibit significant radical scavenging activity and thus act as potential antioxidants (Zhang *et al.* 1994; Abe *et al.* 1999). These reports suggest the potential of saffron as a remedy for major human diseases and provide a strong interest to explore the medicinal and nutritional significance of this valuable spice.

PHYTOCHEMISTRY

Over the past decade, significant progress has been made in evaluating the individual constituents of saffron and their pharmacological properties. Based on the studies from various laboratories, it has been concluded that saffron and extracts of saffron contains three main pharmacologically active metabolites that may be categorized as dyes (crocetin and crocins), bitter principle (picrocrocin) and volatile agents (safranal). Thus the color of saffron is attributed mainly to the degraded carotenoids (crocin and crocetin), and the flavor is derived from the carotenoid oxidation products (mainly safranal and the bitter glucoside picrocrocin). Pfander and Schurtenberger (1982) suggested that the biogenesis of these compounds occurs by bio-oxidative cleavage of zeaxanthin.

Crocins, the water-soluble carotenoids found in the stigma of the saffron are the most important and major compounds of saffron that have been shown to exert significant biological activities. The yellow pigment crocin containing a gentiobiose (disaccharide) group at each end of the molecule and its three other derivatives are the major colour compounds of saffron. The core compound of crocins is the crocetin which is a diterpenoid with a 20-carbon chain dicarboxylic acid. The five major biologically active ingredients namely, the four crocins and crocetin have been quantified by a simple, specific HPLC method (Li *et al.* 1999). Other minor carotenoids are also present in saffron besides the crocins.

Picrocrocin, a monoterpene glycoside is the chemical foremost contributing for the bitter taste of saffron. Picrocrocin [4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde] (**Fig. 1**) is the degradation product of zeaxanthin and the precursor of safranal. During the drying process, for commercial purposes, picrocrocin liberates aglycone (HTCC, $C_{10}H_{16}O_2$) due to the action of the enzyme glucosidase. The aglycone is then transformed to safranal, a terpene aldehyde on dehydration. Safranal [2,6,6-trimethylcyclohexa-1,3-dien-1-carboxaldehyde], which is synthesized via deglucosylation of picrocrocin composes as much 70% of total volatiles and is responsible

for the odour and aroma distinctive of the plant. Thus, a good quality saffron may be said to consist of about 30% of crocins, 5 to 15% of picrocrocin and usually up to 2.5% of volatile compounds (Schmidt *et al.* 2007).

Additionally, 2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one, is one more element considered as powerful contributor to saffron's fragrance despite its being present in a lesser quantity than safranal (Jessie and Krishnakantha 2005). Furthermore, evidences support the presence of proteins, sugars, vitamins (especially riboflavin and thiamin), flavonoids, amino acids, minerals, gums, and pigments including, anthocyanin and lycopene in *C. sativus* (Abdullaev 1993; Ríos *et al.* 1996; Winterhalter and Straubinger 2000; Giaccio 2004).

CURRENT AWARENESS ON PHARMACOLOGY

Although saffron has wide-ranging therapeutic benefits, much research has been focused on its antioxidant, anti mutagenic and anti tumoral properties. A brief appraisal on the promising saffron research, directing its future role as a potential pharmacological source, has been reviewed in this paper.

Anti oxidative activities

Polyunsaturated fatty acids making up the lipid bilayers of cell membranes, expose them at a greater risk to peroxidetion. Free radical reactions in the lipid bilayer have been indicated to result in membrane damage and thereby alteration and impairment of membrane functions (Wiseman 1996) leading to adverse effects including atherosclerosis and cancer (Cook and Samman 1996; Schmidt *et al.* 2007). Reactive oxygen species stimulating oxidative damage to cellular macromolecules have been reported in association with various pathological conditions. Antioxidant therapy has been well documented to protect against such injuries (Love 1999; Gilgun-Sherki *et al.* 2002). Interestingly, saffron has been recommended for its free radical scavenging property that inhibits the lipid peroxidation (Tyler 1975; Halliwell and Gutteridge 1984; Rios *et al.* 1996).

It has been suggested that safranal has an overall protective effect against cerebral ischemia/reperfusion injury-induced oxidative stress in a rat model. It is evident from the conclusions of Magesh *et al.* (2006) that crocetin inhibits lipid peroxidation and increase antioxidant status by enhancing the glutathione activity. Hosseinzadeh and Sadeghnia (2005a) have measured the effect of safranal on lipid peroxidation in terms of malondialdehyde (MDA), a stable metabolite of the free radical-mediated lipid peroxidation cascade. Safranal reversed the increase in the MDA levels confirming its antioxidant role in Ischemia reperfusion injury (IRI). The same group have also evaluated the antioxidant potential of hippocampus homogenate samples following IRI, using ferric reducing antioxidant power (FRAP) assay. Experiments have also revealed that the component(s) of saffron extract inhibit lipid peroxidation in human platelet membranes induced by iron-ascorbic acid system (Jessie and Krishnakantha 2005). The effect of crocin and the aqueous saffron extract against renal IRI has been assessed by measuring the MDA levels and total thiol concentration (Hosseinzadeh *et al.* 2005b). The results have shown that the saffron extract was more potent than crocin which may be attributed to the presence of extensive constituents (crocins, crocetin, dimethyl crocetin and flavonoids) having the potential to quench free radicals.

Studies from our laboratory have shown that aqueous extract of saffron protects antitumor agents induced genetic damage (Premkumar *et al.* 2006) and also inhibits the genotoxin-induced oxidative stress in mice liver and increase the levels of glutathione (GSH) concentration, glutathione *S*-transferase (GST), glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD) activities (Premkumar *et al.* 2001, 2003).

It has been well known that reactive oxygen species (ROS) also cause lipid peroxidation in the sperm cell membrane, impairing the sperm motility and its efficiency to fuse with the oocyte (Bolle *et al.* 2002; Agarwal *et al.* 2008). However antioxidants preserve fatty acids from oxidation, and therefore, have been implicated to play an important role in male fertility (Bolle *et al.* 2002). In view of these, Heidary *et al.* (2008) conducted a minor study to reveal the positive effect of saffron and its constituents, crocetin and dimethyl crocetin, on semen parameters such as motility and morphology (Heidary *et al.* 2008).

Anti-tumor and anti-mutagenic activities

A wide variety of naturally occurring substances including spices (Van Popper 1993) have been shown to inhibit chemical carcinogenesis in animal models (Williams 1984; Boone *et al.* 1990; Winterhalter and Straubinger 2000). One candidate spice, research on whose effect on neoplastic cells has seen a renaissance in the last decade is saffron. A growing body of evidence suggests the chemopreventive effects of saffron and its extracts, both *in vivo* and *in vitro* (Salomi *et al.* 1990, 1991a; Nair *et al.* 1991b, 1994, 1995; Abdullaev *et al.* 2000; Abdullaev 2002; Abdullaev *et al.* 2002; Das *et al.* 2004). Saffron has been quoted as a potential agent for reducing cisplatin-toxic side effects (Fernández *et al.* 2000) including nephrotoxicity. Oral administration of saffron extract have shown marked inhibition of growth of ascite tumors derived from sarcoma-180 (S-180), Ehrlich ascites carcinoma (EAC), Dalton's lymphoma ascites (DLA) in a dose-dependent manner and thereby increased the life span of the drug treated tumor-bearing mice (Nair *et al.* 1991a). Later reports have confirmed that oral administration of saffron extract suppressed the growth of DLA and S-180 tumour cells but did not affect the growth of EAC tumour cells in mice (Nair *et al.* 1992).

Numerous studies have demonstrated antitumor effect of saffron and its constituents on various malignant cells *in vitro*. The differences in sensitivity observed (**Table 1**) can be attributed to the distinct cellular properties or the methods of determination of cytotoxicity. It has been shown that nucleic acid synthesis was inhibited by saffron and its extracts without any profound effect on protein synthesis in tumor cells (Abdullaev and Frenkel 1992b; Nair *et al.* 1992). Evidence also supports the stimulatory effect of saffron extract on non-specific, *in vitro* proliferation of immature and mature lymphocytes and colony formation of normal human lung cells (Abdullaev and Frenkel 1992a; Nair *et al.* 1992).

Saffron extract has shown to be potential Cytotoxic on murine tumor cells (S-180, EAC, DLA), mouse leukemia cells (P388), Osteo and ovarian sarcoma, human cervical cancer (HeLa), adeno-carcinoma (A549), lung cancer (WI-38VA), human rhabdomyosarcoma (A-204), liver cancer (HepG-2), and colon cancer (SW-480) at IC_{50} value ranged as 7-200 µg/ml. Bioactive metabolites of saffron such as crocin, crocetin and picrocrocin also shown lower IC_{50} concentration on human leukemia cells. Other metabolites and extracts of saffron such as β-carotene, safranal, all-*trans* retinoic acid, saffron proteoglycan, saffron corm callus extract, glucoconjugate from saffron corms are cytotoxic to cervical cancer, fibro-sarcoma and breast cancer (Abdullaev *et al.* 2002).

Significantly, crocetin is the most studied saffron component that has been consistently linked to low the risk of cancer (Magesh *et al.* 2006). It has been reported that crocin decreased tumor growth (adenocarcinoma of the colon) and enhanced the survival in female rats without any significant effects in male animals, suggesting the influence of hormones on drug action (García-Olmo *et al.* 1999). As well the non mutagenic property of crocin and dimethyl-crocetin has been indicated using the Ames assay.

Antitumor activity of crocetin has been studied by Magesh *et al.* (2006). In their experiments, the effect of crocetin against tumor progression in lung-cancer bearing mice has been elaborately considered with reference to tumour incidence, antioxidant enzymes, marker enzymes and histo-

Table 1 Cytotoxic effect of saffron and its components on human malignant cells.

Agents	Cells	References
Saffron extract	HeLa; A549; WI-38VA	Abdullaev *et al.* 1992a, 1992b
Saffron extract	A-204; HEPG-2; SW-480	Abdullaev *et al.* 2003
Saffron extract, picrocrocin, safranal	HeLa	Escribino *et al.* 1996
Saffron corm callus extract and saffron proteoglycan	HeLa, fibrosarcoma, and breast carcinoma	Escribino *et al.* 1999a, 1999b, 1999c; Escribino *et al.* 2000; Fernández *et al.* 2000
Glucoconjugate from saffron corms	Tobacco BY-2 cells, protoplasts	Fernández *et al.* 2000
Crocin	HL-60; K562; HeLa; and HT-29, DHD/K12-PROb	Morjani *et al.* 1990; Tarantilis *et al.* 1994; Escribino *et al.* 1996; García-Olmo *et al.* 1999
Crocetin, dimethyl crocetin	HL-60; K562	Morjani *et al.* 1990; Tarantilis *et al.* 1994
β-Carotene	K562	Morjani *et al.* 1990
Saffron extract	Glucose-induced PC12 cells	Mousavi *et al.* 2010
Saffron extract	S-180; EAC; DLA; P388 osteosarcoma; ovarian sarcoma	Nair *et al.* 1991a; Salomi *et al.* 1991b; Nair *et al.* 1992, 1995
All-*trans* retinoic acid	HL-60	Tarantilis *et al.* 1994

pathological analysis. The decrease in the activities of the marker enzymes on treatment with crocetin has suggested its antineoplastic property that offers protection against abnormal cell growth (Verma and Bordia 1998). Another study has indicated the inhibitory action of crocetin at non toxic doses on the genotoxic effect and neoplastic transformation induced by benzo(a)pyrene in C3H1OT1/2 cells (Chang *et al.* 1996). Both these results have demonstrated that crocetin does not exhibit any genotoxicity. Two studies (Morjani *et al.* 1990; Tarantilis *et al.* 1995), have indicted the cytotoxic activity of crocetin on tumor cells. In contrast, another study has shown that crocetin did not exhibit any cytotoxic effect (Escribano *et al.* 1996). Previously, work carried out by Abdullaev's laboratory has also demonstrated that crocetin possessed no cytotoxic effect on colony formation of different tumor cells, but had an inhibitory effect on DNA, RNA, and protein synthesis (Abdullaev 1994). Reports have also confirmed that saffron extracts in combination with eminent antitumor agents such as selenium compounds caused a more effective inhibition of colony formation and nucleic acid synthesis (Abdullaev and González de Mejia 1995-1996). The strong anti tumor activity of crocetin is thus well established making it a candidate compound that can be tested for its effects against several other cancer systems.

Apart from the main components of saffron stigma, a novel glycoconjugate from corms and callus of saffron has been shown to possess cytotoxic activity against different tumor cells derived from fibrosarcoma, cervical epithelioid carcinoma, and breast carcinoma (Escribano *et al.* 1999b, 2000). The glycoconjugate has shown about eight times more cytotoxic for malignant cells causing plasma membrane damage in these cells. However, DNA fragmentation analysis has indicated the absence of apoptosis mediated cell death (Escribano *et al.* 1999c, 2000; Fernández *et al.* 2000).

The antimutagenic, comutagenic and cytotoxic effects of saffron and its ingredients has been assessed using the Salmonella test system, *in vitro* colony formation assay and four different cultured human normal (CCD-18Lu) and malignant (HeLa, A-204 and HepG2) cells (Abdullaev *et al.* 2003). In the *Salmonella* test system, saffron has exhibited non-mutagenic and non-antimutagenic activity against BP-induced mutagenicity. A dose-dependent co-mutagenic effect on 2-AA-induced mutagenicity has also been observed that is reported to be due to the saffron component, safranal. Saffron has displayed inhibition of colony formation only against human malignant cells and cytotoxicity against *in vitro* tumor cells.

Anti-cancer activity

Several reports on the anticarcinogenic effects of saffron have been put forward in the last decade (Salomi *et al.* 1991b; Dufresne *et al.* 1997). Ethanolic extract of saffron have shown to exert significant inhibitory action on colony formation and DNA and RNA synthesis in Hela cells (cervix epitheloid carcinoma cells (Abdullaev and Frenkel 1992b). Another study performed by the same group on A549 cells (lung adenocarcinoma cells), WI-38 (normal lung fibroblast-like cells), and VA-13 (WI-38 cells which were transformed by SV-40 viruses) has presented higher sensivity to the inhibitory action of saffron in comparison with normal counterparts (Abdullaev and Frenkel 1992a). *In vitro* inhibitory action of saffron stigma aqueous extract on the proliferation of human TCC and mouse L929 cells in a dose dependent manner has been studied (Isa 1992).

A study conducted for testing the efficacy of *C. sativus* extract and its major component crocin against three colorectal cancer cell lines (HCT-116, SW-480, and HT-29) has demonstrated significant inhibition on the cancerous cell growth (Aung *et al.* 2007). Various conclusions derived from this study include that at cancer cell inhibitory concentrations, the extract did not affect non-cancer cells. Further comparison has shown that HCT-116 cell line had a higher sensitivity to saffron extract and crocin than other two cells, SW-480 and HT-29. As the HCT-116 cells are p53 wild-type cells, the strong reaction of crocin and saffron extract on them suggests that p53 activity may be associated to the compounds present, exerting anticancer effects.

A great deal of interest has been shown for the elucidation of possible mechanisms for the tumoricidal and anti cancer activities of saffron compounds. One proposed mechanism for the antitumor or anticarcinogenic action of saffron is its inhibitory effect on cellular DNA and RNA synthesis, but not on protein synthesis (Abdullaev and Frenkel 1992b; Abdullaev 1994; Nair *et al.* 1994, 1995; Abdullaev and González de Mejia 1995-1996). Inhibition of free radical chain reactions, indicating their antioxidant properties may be another possible mechanism for antitumor and anticancer activities (Tyler 1975; Palozza and Krinsky 1992; Nair *et al.* 1994, 1995; Dufresne *et al.* 1997; Abdullaev and Frenkel 1999; Li *et al.* 1999; Molnar *et al.* 2000; Violette *et al.* 2002). Metabolic conversion of naturally occurring carotenoids to retinoids is a third proposed mechanism by which the saffron exerts its antitumor effect (Tarantilis *et al.* 1995; Dufresne *et al.* 1997), but it has been recently reported this conversion is not a necessary condition for the anticancer activity (Smith 1998). And finally the cytotoxic effect of saffron is said be related to the interaction between carotenoids and topoisomerase II (Isa 1992; Nair *et al.* 1995; Smith 1998). The lectin content (Oda and Tatsumi 1993; Escribano *et al.* 2000b) can also be suggested for antitumor activity of saffron in addition to the reports suggesting the inhibitory effect of saffron on various cellular enzymes and their functions (Nair *et al.* 1992; Abdullaev and González de Mejia 1997; El Daly 1998; Kubo and Kinst-Hori 1999). Saffron cytotoxicity can also be attributed to apoptosis (Morjani *et al.* 1990) and a sharp increase in the level of intracellular SH compounds. Research on PC-12 cells, crocin inhibited cell growth by its effects on tumor necrosis factor alpha (Ochiai *et al.* 2004).

The exact mechanisms of the tumoricidal and anti-cancer effects of saffron, however, need to be established on a strong molecular platform to facilitate its clinical usage.

OTHER BIOLOGICAL ACTIVITIES OF SAFFRON

Anti-depressive effects

Aqueous and ethanolic extracts of saffron and its constituents safranal and crocin have shown antidepressant effects in mice on intraperitoneal administration, using the forced swimming test. Safranal and crocin are reported to contribute to the antidepressant effect. The possible mechanisms proposed for safranal and crocin seems to be via inhibition of serotonin reuptake and inhibition of dopamine and norepinephrine re-uptake, respectively (Karimi *et al.* 2001; Hosseinzadeh *et al.* 2004). Additionally, crocin has been found to antagonize ethanol-induced depression via NMDA-receptor, *in vitro* (Abe *et al.* 1998).

Antinociceptive and anti-inflammatory activities

An *in vitro* study (Hosseinzadeh and Younesi 2002) indicates that the antinociceptive and anti-inflammatory activity of aqueous and ethanolic extracts of petals and stigma of *C. sativus*. Antinociceptive studies including the hot-plate test (55 ± 0.2°C) and writhing tests assessed on saffron pre-treated mice have revealed interesting results suggesting the anti nociceptive effect of the stigma and petal extracts.

Anti-inflammatory studies carried out with xylene-induced inflammation (ear edema) in mice have shown the absence of increase in weight signifying the anti-inflammatory activity of the stigma and petal extracts (Hosseinzadeh and Younesi 2002). Another study with formalin induced inflammation conducted with six groups of rats treated with the extracts has also shown negative results for inflammation thus proving the anti-inflammatory effects of *C. sativus* stigma and petal extracts. The significant antinociceptive and anti-inflammatory effects of the petal extracts can be attributed to the flavonoid content, tannins and anthocyanins. Recent studies have recognized crocins and *Crocus* glycosides, to exhibit anti-inflammatory effect in some models of inflammation.

Effect on learning abilities and memory

The oral administration of saffron extract has showed distinct improvement of the ethanol pre-damaged memory of mice, although no effect on learning abilities in the passive avoidance test has observed. This effect is attributed to crocin, which has been proved to improve cognitive functions in animals whose memory had been experimentally impaired (Sugiura *et al.* 1994, 1995).

FUTURE PERSPECTIVES

Chemoprevention involves pharmacological intervention with specialised agents, both synthetic and natural, to reverse, repress, or prevent human cancers. The key crisis in employing synthetic chemopreventives in cancer treatment is the potential toxicity of these drugs to surrounding healthy cells. A possible approach to solve this problem is to pursue the 'back-to-nature' trend and explore the potential of plant and plant based dietary products against tumorigenesis. Evidences have shown that saffron and its components can affect carcinogenesis and is currently being studied at length as the most promising cancer chemopreventive agents. With the increasing need/requirement for natural chemopreventive agents for treating cancer and also various other diseases worldwide, a new strategy must be devised for the assessment of drug efficacy, effectiveness and toxicity of the medicinal compounds in saffron for optimization of its therapeutic potential.

Comprehensive studies determining biologically active components of saffron and defining the mechanism(s) involved in cancer chemoprevention need to be conducted in order to promote the use of saffron in cancer therapy. The inhibitory effect of saffron extract in combination with other synthetic drugs can also be exploited to provide evidence for a combinational therapy against tumours. Also, since current research has proved the anti-tumoural properties of saffron extracts, both *in vitro* and *in vivo*, it is necessary that future research needs to focus on performing human studies and clinical trials to define efficacy of saffron in cancer treatment and prevention.

In addition to the discovery of core components and their mechanisms of action, studies to investigate suitable drug delivery systems and to increase the effectiveness of the drug may also be conducted. The application of nanoparticles (NPs) to facilitate targeted delivery of the saffron components drug and their slow release is yet another interesting area for future research. However, the scarcity, sterility and expense in obtaining saffron may impede its usage in chemo prevention. Hence, considerable agronomic research to develop new production technologies of the plant may also be anticipated. Identification of novel methods to increase the production of therapeutic components in saffron by biotechnological methods such as enzyme conversion, callus induction and elicitation with natural elements can also be a part of such research.

Thus these various aspects afford a sincere hope that saffron will contribute to public health in the new century and also lay the platform for the emergence of a new scientific discipline that can be referred as *saffronology*.

SUMMARY

Saffron, the vernacular name for *C. sativus*, is the most precious and most expensive spice in the world. From time immemorial the spice has been cited in many indigenous and home-grown systems of health care for treatment of a wide-range of diseases and disorders. Modern pharmacological research have been intended to investigate the chemical constituents and their biological activity to confirm these traditional claims and they have confirmed the antioxidant, anti tumor and anti-depressant properties of the plant. Still there are many compounds in saffron with therapeutic potential in their unexplored fold. If future research is focused on exploring these potential compounds and developing the same into rational phytomedicines, saffron and its constituents will certainly serve as alternative anticancer agents, which alone and in combination with other synthetic drugs, may aid in the prevention and the treatment of existing and new forms of cancer. If future research is focused on unraveling these potential compounds and developing the same into rational phytomedicines, saffron is sure to become an important and integral part of therapeutic medicinal field leading to the development of safe and efficacious anti-cancer therapy. If future research is focused on unraveling these potential compounds and developing the same into rational phytomedicines, saffron will certainly become an important source and integral part of chemopreventive and alternative medicine.

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How to reference: Author name(s) (2010) Title of chapter. In: Husaini AM (Ed) Saffron. Global Science Books, UK, pp. X-XX

Saffron (*Crocus sativus* Kashmirianus) Cultivation in Kashmir: Practices and Problems

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ABSTRACT

The Kashmir valley is well known for quality saffron and represents one of the major saffron-producing areas of the world, dating back to 750 AD. However, in the last decade production and productivity of this crop has shown a declining trend. This paper highlights the practices followed in saffron cultivation in Kashmir and discusses different factors responsible for the decline in saffron production. It also stresses the need for using quality planting materials, a sprinkler irrigation system, pest and disease control measures and an efficient marketing system for increased profitability. Pressure due to increased urbanization on land on which saffron grows and clandestine saffron smuggling are contributing towards the decline of the saffron industry, and these have also been discussed.

Keywords: corm, disease, phenology, planting cycle, post-harvest handling, urbanization

CONTENTS

INTRODUCTION

Jammu and Kashmir (J&K) state is located between 32°17′ to 36°58′ North (latitude) and 73°26′ to 80°30′ East (longitude), encompassing the Western Himalayas and the Karakorum mountains. One of the largest states of the Indian Union, J&K covers an area of 2,22,236 km^2 and includes, besides the famous Kashmir valley, the area of Jammu, Ladakh, Baltistan, Gligit, Hunza and Nagar (**Fig. 1A**). Amongst these, the Kashmir valley represents one of the major saffron (*Crocus sativus* Kashmirianus) (**Fig. 1B**)-growing areas of the world. The time at which saffron was introduced to Kashmir is not precisely known, although evidence from '*Rajatarangini*', written by a 12th century poet-historian (Kalhana), indicates its presence in Kashmir even before the reign of King *Lalitaditya* in 750 AD. This "golden" spice is known as '*Kum Kum*' and '*Kesar*' in Sanskrit, and '*Koung*' in Kashmiri language.

Even though successful attempts to grow saffron in other areas of India such as Uttar Pradesh and Himachal Pradesh have been reported (Dhar and Mir 1997) as well as in other parts of J&K state like Kargil (Munshi *et al.* 2002), almost all saffron production is actually limited to Kashmir. In Kashmir, saffron is grown on uplands (termed in the local dialect as 'Karewas'), which are lacustrine deposits located at an altitude of 1585 to 1677 m above mean sea level (amsl), under temperate climatic conditions (Kanth *et al.* 2008). The soils are heavy textured with silty clay loam

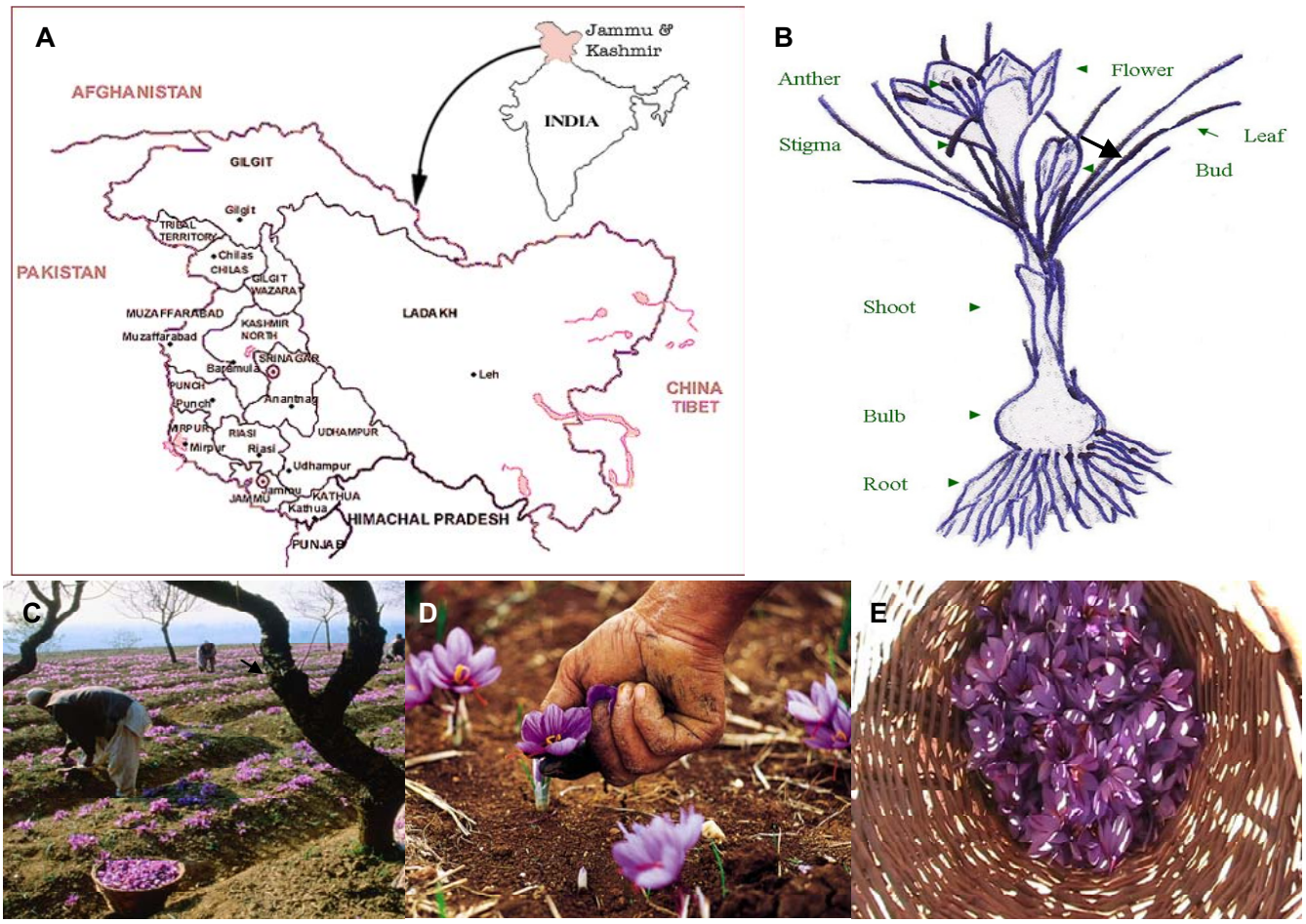

Fig. 1 Saffron in Kashmir. (**A**) The state of Jammu and Kashmir. (**B**) Sketch of *Crocus sativus* Kashmirianus plant. (**C**) Field laid into rectangular strips and alley cropping with almond. (**D**) Saffron flowers harvested by hand picking. (**E**) Flowers collected in a wicker basket or *Tokri*.

as the predominant texture in upper horizons and silty clay in lower horizons. These soils are alfisols and are well drained. The soils are calcareous in nature with average organic carbon and calcium carbonate contents of 0.35 and 4.61%, respectively. The soil is slightly alkaline with pH ranging from 6.3 to 8.3 and with electrical conductivity between 0.09 and 0.30 dsm^{-1} (Nazir *et al.* 1996; Ganai *et al.* 2000; Ganai 2002). Higher yields coincide with higher pH values (Shinde *et al.* 1984).

The total area under this crop in the State in 2007-08 was 3,110 ha with an annual production of 5.06 t and productivity of 1.62 kg ha^{-1} while almost a decade back in 1996-97 the area recorded was 5,707 ha with an annual production of 15.95 t and productivity of 2.79 kg ha^{-1} (Anonymous 2009). This shows a decrease of 83% in area, 215% in production and 72% in productivity of saffron in one decade in Kashmir. The lowest productivity of 1.57 kg ha^{-1} was recorded in 2003-04 due to an acute drought from 1999-2003 (Anonymous 2008, 2009) (**Table 1**). According to the data available for 1990-96 (not shown) the area of saffron cultivation in Kashmir varied in a narrow range of 4036 to 4496 ha, with more or less constant annual production (13.0-14.1 t) and productivity (2.90-3.21 kg ha^{-1}).

In the present paper we discuss cultivation practices of saffron followed in Kashmir, highlighting the major constraints that limit its production and productivity while also suggesting some ameliorative measures for its sustainable production.

Table 1 Year-wise trends in area, production, productivity of saffron in Kashmir.

Year	Area (ha)	Production (t)	No. of rainy days	Total rainfall (mm) [ss]	Yield (kg ha^{-1}) [ss]
*1996-1997	5707	15.95	49	583.7	2.79
#1997-1998	NA	NA	37	548.8	NA
*1998-1999	4116	12.88	50	387.4	3.12
*1999-2000	3997	7.65	52	399.7	1.91
*2000-2001	2831	3.59	49	518.5	1.26
[$]2001-2002	2880	6.52	NA	NA	2.26
[$]2002-2003	2742	5.15	72	884.0	1.87
[$]2003-2004	3075	4.83	63	635.2	1.57
[$]2004-2005	2989	8.85	72	680.8	2.96
[$]2005-2006	2928	4.85	89	887.6	1.65
[$]2006-2007	2436	9.13	3.74	501.8	3.74
[$]2007-2008	3110	5.06	NA	NA	1.62

Data not available (NA)
*Source: Planning Department, J&K Government, and Directorates of Agriculture, Kashmir and Jammu Divisions
[$] Source: Economic Survey 2008-09, J&K Government
[ss] Source: Regional Metrological Centre, Srinagar, J&K; these are the figures for Srinagar district only and do not pertain to the whole area under saffron cultivation, as it is not available.

PHENOLOGY OF SAFFRON

Phenology, the study of development of a plant in relation to its environment, is important for understanding the basis for cultural operations in a crop. The phenology of saffron in Kashmir, as described by Kanth *et al.* (2008), occurs in three phases.

The first, generative phase starts with the onset of cold weather in fall and is an important stage for growers. Irrigation in late summer and early autumn is beneficial for this phase, as the physiological processes start well before apparent flower emergence. In Kashmir, this stage is recorded in mid-October to the first week of November and covers about 20-25 days.

The second, vegetative phase is the longest period in the life cycle of saffron and starts immediately after the flowering stage. At this stage leaves develop and provide necessary nutrients for corms. This stage can start early by an early rainfall or early irrigation, and leaves may emerge simultaneously with flowers. This phase lasts for at least 6 months (November to April) in Kashmir.

The third, dormant phase starts with leaf withering and senescence in the spring and ends with the first irrigation in late summer and early autumn. This period is considered by growers as the rest period. In Kashmir this stage is observed from April to September.

CULTURAL AND POST-HARVEST PRACTICES FOR SAFFRON PRODUCTION

In Kashmir, in spring (March-April), the field is ploughed either using a plough or tractor twice with an interval of about 20 days. In August, levelling and hoeing operations are carried out and fields are pulverized 3-4 times. Well-decomposed farm manure (15-20 t ha^{-1}) is applied by some progressive farmers and thoroughly mixed into the soil before the last tillage operation (Mir 1992; Munshi *et al.* 2002). However, in addition to the application of farm manure, chemical fertilizers supplying nitrogen (40 kg ha^{-1}), P_2O_5 (50 kg ha^{-1}) and K_2O (30 kg ha^{-1}) are recommended to restore and sustain soil fertility, although in actual practice the farmers do not apply these chemical fertilizers (Mir 1992; Munshi *et al.* 2002).

The field is laid out into rectangular strips (1.5-2 m wide and 2-3 m long) with drainage channels (30 cm wide and 20 cm deep) on both sides (**Fig. 1C**). The corms are sown at depth of 12-15 cm in these raised beds with 10×20 cm spacing (inter-corm and inter-row). The most suitable time for sowing the corms is from the last week of August till mid September (Mir 1992; Munshi *et al.* 2002).

The total rainfall during the saffron growing period is usually sufficient, but its distribution is not regular, and usually saffron faces some water stress. If rains are received at sprouting and pre-flowering stages, the flowering is optimum that year and saffron yields are good (Anonymous 1988). Any major drop in ambient temperature or un-seasonal rains during October causes serious damage to flowering, leading to heavy reduction in saffron yield. Flowering occurs between mid-October to mid-November every year, mostly in 3 flushes in a normal season.

In Kashmir flowers are picked by hand (**Fig. 1D**). This is done early in the morning mostly by female members of the family and to a limited extent by hired labor. The flowers are collected in wicker baskets, called in local language 'Tokri' (**Fig. 1E**). These are then taken to sheds or houses for separating the stigma and other flower parts. This is a tedious job and generally takes the whole night or more for each day's harvest. The separated flower parts are then dried in the shade for 2-3 days provided the days are sunny, otherwise drying is extended by a day or two.

The process of drying has a great effect on the quality and worth of the final product. In Kashmir, saffron is dried under shade or sometimes under direct sunlight. They are left there until the moisture evaporates, and only 10-11% water content remains in the stigma. The dried material (stigma alone called *'Mongra'* or stigmas attached with parts of the style, called *'Laccha'*) is packed mostly in poly bags and stored at room temperature until the farmer gets the desired price. The storage period varies from 25 days to 6 months (Mir 2002).

In some areas of Kashmir saffron is cultivated as an alley crop with almond trees (**Fig. 1B**). Rotation of saffron fields after a planting cycle of about 15 years is a common practice in Kashmir. Saffron fields are either kept fallow or rotated with linseed, maize, oats for a period of 2-3 years. This helps in control of pests and diseases, and restoration of soil fertility (Nehvi *et al*. 2008a).

MAJOR CONSTRAINTS FACING SAFFRON PRODUCTION IN KASHMIR

Non-availability of good quality corms

The saffron plant, being triploid, fails to set seeds, and thus is propagated vegetatively through corms. Well developed and large disease-free corms are an essential component for sustainable saffron production. As such, sorting of corms on the basis of weight and size is an important pre-requisite for higher productivity in saffron. A flowering corm contains 10-12 buds and each sprouting bud produces a cormel. Pandy *et al.* (1979) found that larger corms produced more leaves and flowers. This kind of positive relationship between corm size and number of flowers per corm and saffron yield is well established (Dhar 1991; DeMastro and Ruta 1993; McGimpsey *et al.* 1997; Omidbeigi *et al.* 2003). Small corms do not have the potential to produce flowers in the first year (Sadeghi 1983), while corms larger than 2.5 cm in diameter only flower (DeMastro and Ruta 1993). In addition, the weight of corms is also very variable and ranges between 1 and 20 g (Behnia *et al*. 1999). Corms weighing around 2 g do not flower and up to 8 g show very limited potential while corms weighing more than 10 g have the capacity to flower in the same year and are necessary for higher yield (Sadeghi 1993).

In Kashmir, corms with diameter greater than 2 cm and 10 g in weight are recommended for sowing, but due to non-availability of standard corms farmers generally plant substandard corms of smaller size and weight (Nehvi *et al.* 2004). In addition only one corm per hill is sown which leads to further low plant population and low productivity (Hassan and Shah 2002; Munshi *et al.* 2002). Due to the repeated use of the same substandard seed material year after year, the productivity of saffron in Kashmir is grossly compromised. Even when seed corms become available when some farmers dig out their fields for new planting the corms are sold at a high price (at Rs. 20000-40000 t^{-1}), which is unaffordable for small and marginal farmers.

Seed rate and planting cycle aberrations

In Kashmir, saffron is grown using corms of diverse sizes at a low seed rate of 1.5-2 t ha^{-1}, with the exception of a few progressive farmers who use about 4 t ha^{-1}. Due to the non-availability of seed corms for new planting in sufficient quantities small and marginal farmers use a seed rate which is about 3 times less than the recommended 5 t ha^{-1} (Nehvi *et al.* 2007a).

According to a comparative study by Kafi and Showket (2007) the number of corms sown $unit^{-1}$ of land in Kashmir is much lower than Khorasan (Iran). For instance, 40-50 corms are sown in 1 m^2 of saffron farm in Kashmir compared to 150-250 corms m^{-2} in Khorasan. In addition, only a single corm $hill^{-1}$ is sown in Kashmir (at a depth of 10-15 cm) in contrast to 3-15 corms $hill^{-1}$ (at 15-20 cm depth) in Khorasan. This could be the main reason why the farmers of Kashmir harvest negligible saffron flowers in the first year.

Studies have revealed that under a high plant population, 4-year-old fields give maximum saffron yield (14 kg ha^{-1}), whereas under low plant population maximum saffron yield

is achieved at 6 years of age (13 kg ha^{-1}). There is a sharp decline in yield (< 3 kg ha^{-1}) when fields are 13 years old (Koul and Farooq 1984; Negbi 1999). In Kashmir, corms are planted under low population density (1.5-2 t ha^{-1}) and the average age of saffron fields is more than 15 years, therefore an important factor for low productivity. A well developed mother corm produces 15-20 cormels by the end of the 4th year. Studies on the planting cycle in Kashmir revealed maximum recovery of corm yield (16.1 t ha^{-1}) and saffron yield (4.8 kg ha^{-1}) after 4 years, which clearly suggests that saffron fields should be rejuvenated after every 4-5 years (Nehvi *et al.* 2004, 2008b). However, due to non-availability and high cost, the mother corms are retained in fields for many years even at the expense of declining productivity. Under larger planting cycles the overcrowded daughter cormels produce large numbers of contractile roots and later weaker cormels which die and decay due to lower availability of nutrients. A shorter planting cycle of 4-5 years, combined with appropriate planting depth of the mother corms, is most appropriate to harvest a higher proportion of large-sized corms.

Poor soil fertility

Growing saffron year after year without the supply of nutrients through organic manures and/or chemical fertilizers has drastically reduced the fertility of the soils in Kashmir saffron fields. These soils have become deficient in organic carbon as well as in micro-nutrients. Consequently, the size and vigor of the corms produced each season is reduced, directly affecting the crop stand and flowering potential of plants. The application of organic manures in recommended doses helps to enrich the soil with an adequate quantity of essential nutrients, improves soil health, water use efficiency and better growth of saffron shoots. Production of higher vegetative biomass (more leaves, longer leaf length and higher dry matter content of aerial shoots per corm) in turn results in the production of better daughter corms. The application of farm yard manure (FYM) (17.5 t ha^{-1}) in combination with inorganic fertilizers N: P_2O_5: K_2O (30: 20: 15 kg ha^{-1}) to quality saffron corms (>10 g) planted at a density of 0.5 million corms ha^{-1} resulted in corm yield of 16.5 t ha^{-1}, while in the control only 6.20 t ha^{-1} was recorded. The proportion of good quality corms with an average weight of above 10 g was 52.6% (Nehvi 2004). The application of FYM and chemical fertilizers also results in an increase in saffron yield. Nehvi (2004) reported an annual saffron yield of 1.66 kg ha^{-1} with the application of FYM (17.5 t ha^{-1}) + N: P_2O_5: K_2O (30: 20: 15 kg ha^{-1}), which is 62.7% higher than the control. Similarly, Kirmani (2010) recently reported saffron yield of 3.5-3.6 kg ha^{-1} when either FYM (60 t ha^{-1}) or nitrogen (90 kg ha^{-1}) was applied, which was 57% higher than the control; the crocin content was 12.35% while in the control it was 10.79%.

In a separate experiment three different doses of FYM (15, 20 and 25 t ha^{-1}) were tested in a saffron field in Kashmir, with three different corm sizes, viz. < 10 g, 10-15 g and > 15 g, planted at three different densities (0.5, 0.65 and 0.80 million ha^{-1}). The application of FYM at 25 t ha^{-1} using heavier corms (>15 g) at a density of 0.5 million ha^{-1} (10 × 20 cm, i.e., 10 cm inter-corm distance, and 20 cm inter-row distance) resulted in the production of quality planting material with a maximum proportion of flower-producing corms (Verma *et al.* 2008). The treatment resulted in maximum corm yield of 15.3 t ha^{-1} under a biannual planting cycle, with a yield advantage of 63.5% over the control. The proportion of flower-producing corms was 57.3%. Highest stigma length of 3.8 cm, style length of 3.5 cm, perianth size of 6.9 × 5.7 cm, highest number of leaves per mother corm (45.3) and maximum length of leaves (19.0 cm) were also recorded in this treatment. The results show that the application of FYM and corm weight are the most important variables for higher production of quality planting material whereas increased planting density (> 0.65 million ha^{-1}) showed a detrimental effect on corm multiplication and their further development.

Lack of assured irrigation

Saffron fields in Kashmir are entirely rain-fed. If rains fail, the crop also fails. Although water requirement is low for saffron, water stress affects yield, growth and development (Hassan and Shah 2002; Munshi *et al.* 2002). Srivastava (1963) reported that areas receiving 100-150 cm of well distributed rainfall with snow in winter are suitable for saffron cultivation, and rains in September are essential for meeting the water requirement of corms for good flower yields. Rainfall of 100-150 mm is considered essential during the pre-flowering stage (Kamili *et al.* 2007). However, due to climate change in the last several years the weather has become quite erratic and rains are either scanty or distribution is irregular, thus adversely affecting flowering in saffron. The State of Kashmir faced an acute drought in 1999-2003 (Alam 2007), and during this period productivity was reduced from about 3.12 kg ha^{-1} to 1.57 kg ha^{-1} (**Table 1**). However, favorable rainfall during 2004-05 improved saffron productivity to 2.96 kg ha^{-1}.

The campaign of improving productivity of saffron would not bear the desired fruit unless facilities for assured irrigation are created, at least at pre-sprouting and pre-flowering stages. Irrigation facilitates quick activation of buds leading to corm sprouting and flower initiation. According to Nehvi (2004) and Nehvi and Makhdoomi (2007b), the saffron crop requires 10 irrigations, and should be sprinkler irrigated at 70 m^3 ha^{-1} at an interval of 7 days at the sprouting stage (25th August to 15th September) followed by 3 irrigations at the post-flowering stage (8th November to 30th November) at weekly intervals. In a separate study, Aga *et al.* (2008) recommended 5 irrigations, each on the 20th August, 1st, 10th, 20th and 30th September. Despite all these recommendations not much effort is being made by Government agencies to incentivise farmers to use irrigation in saffron fields.

Poor pest and disease management

The intensive cultivation and monoculture of saffron in saffron-growing belts of the Kashmir valley, together with the continual use of diseased material has resulted in the frequent occurrence of corm rot diseases caused by pathogens such as *Rhizoctonia crocorum*, *Phoma crocophila* (Madan *et al.* 1967), *Fusarium moniliforme* var. *intermedium*, a non-sporulating basidiomycetous fungus (Dhar 1992), *Macrophomina phaseolina* (Thakur *et al.* 1992), *Fusarium oxysporum*, *F. solani*, *F. pallidoroseum*, *F. equiseti*, *Mucor* sp., *Penicillium* sp. (Wani 2004; Ahmad and Sagar 2006) and *Sclerotium rolfsii* (Kalha *et al.* 2007). Of these diseases, corm rot of saffron caused by *F. oxysporum* and *F. solani* is considered to be most destructive in Kashmir (Wani 2004; Ahmad and Sagar 2006). These infections generally take place through the injury of corms. Infected corms possess dark-brown sunken and irregular patches below the corm scales, mostly near root and bud regions. In severe cases the entire corm turns into a black powdery mass. The foliage of infected corms shows symptoms of 'die-back' (Ghani 2002). The disease is quite widespread and causes loss of a considerable proportion of the produce every year. Different groups have reported different figures for corm rot incidence in different parts of Kashmir. Rekhi *et al.* (1990) recorded that the disease incidence among farmers in the Kashmir valley was 98% and Dhar (1992) observed that although none of the saffron-growing areas in the Kashmir valley were free from the disease (100% disease incidence), its severity (disease severity) was more in 6.7-15.2% of areas. Nehvi (2003), however, reported the incidence of corm rot as 46% in traditional saffron-growing areas, while Ghani (2002) put the figures at 11.6-21.6% for Pampore (traditional saffron belt). All these studies point to the severity of the problem, which may be due to built-up of inoculum over longer planting cycles, common in Kashmir.

For controlling saffron corm rot, the corms to be planted should be put in a fungicidal solution containing Mancozeb 75WP (0.3%), Carbendazim 50WP (0.1%) for 5-10 min, and then spread on a cloth and allowed to dry in shade for another 10-15 min (Ghani 2002). In a separate study where six fungicides, viz. Blitox (copper-oxychloride), Indofil M-45 (mancozeb), Difolatoan (captafol), Folpat (captafol), Bavistin (carbendazim) and Tecto (thiobendazole) were evaluated, Bavistin and Tecto (0.2% each) as a dip or drench gave complete disease control (Sud *et al.* 1999). In a pot experiment, Ahmad and Sagar (2007) observed that corm treatment with Carbendazim 50WP (0.2%) or Myclobutanil (10WP) (0.2%) proved most effective in reducing the corm rot severity to 7.4 and 5.2%, respectively when corms were dipped overnight in fungicidal suspension, compared to 46.7% in untreated corms.

Besides the damage caused by corm rot, plant parasitic nematodes of many species infesting saffron-growing soil cause damage to corms by sucking the sap. Zaki and Mantoo (2008) reported the percentage infestation at the Konibal area of Pampore as *Helicotylenchus vulgaris* (16.6%), *Pratylenchus thrornei* (8.8%), *Tylenchus* sp. (13.0%), *Tylenchorynchus* sp. (10.7%), *Xiphinema* sp. (14.6%), *Aphelenchus avenae* (5.8%), and *Hemicriconemoides* sp. (3.2%). The sap sucking causes necrosis in roots and predisposes saffron corms to corm rot, causing heavy production losses. Despite this, no systematic control measures are presently being adopted by farmers (Ghani 2002). Application of Chlorpyriphos 10G (at 1000 g a.i. ha^{-1}) or Fenvalerate 0.4% (at 120 g a.i. ha^{-1}) as soil treatment effectively reduces the pest population (Zaki and Mantoo 2008). An ecologically sound solution would be to identify efficient biological control agents. For example, antibiotic-producing *Pseudomonas* strains relevant to biocontrol, such as superior root colonizing ability or higher antibiotic production need to be identified from Kashmir saffron fields.

Poor weed management

Saffron in Kashmir begins its vegetative growth around October-November, which then lasts until April. Thereafter, saffron fields remain vacant due to the dormant phase from April to September. According to Pir *et al.* (2008), the long period from April to September provides an open space for weeds in saffron fields, which gain a monopoly to spread over the entire fields without any resistance that would otherwise be encountered in the presence of the crop. In addition, the saffron plant, being short with narrow upright foliage with little lateral spread offers very little competition to the weeds. The major weeds found in saffron fields of Kashmir include *Euphorbia helioscopia*, *Papaver rhoae*, *Lepidium virginicum*, *Salvia moorcroftiana*, *Chonspora tanella*, *Galium tricorne*, *Tulipa stellata*, *Erodium cicutarium*, *Lithospermum arvense*, *Ranunculus arvensis*, *Medicago lupilina*, *Filago arvense*, *Poa bulbosa*, *Crepis saneta*, *Descurainea Sophia*, *Polygonum aviculare*, *Chenopodium album*, among others (Pir *et al.* 2008). Despite the presence of these weeds no weed management practices are followed by saffron growers except for harvesting of some weeds as fodder by farm women in May, and cattle grazing by some farmers in August. Herbicides have yet to find a place in weed management of saffron in Kashmir. Norouzzadeh and Delghadi (2006) in Iran reported that Ioxynil (750 g a.i. ha^{-1}) and Tribenoronmethyl (18.75 g a.i. ha^{-1}), when sprayed at the 6-8 leafy stage of weeds after saffron harvest, were highly efficient in controlling weeds. In autumn trials, weed control by Ethalfloralin (1320 g a.i. ha^{-1}) and Trifloralin (960 g a.i. ha^{-1}), when applied at pre-emergence and before saffron flowering, was also found to be promising but caused yield loss. The application of Metribuzin (560 g a.i. ha^{-1}) in spring or autumn controlled weeds to a large extent without any injury to saffron.

Weed-infested fields have become a breeding place for rodents, too. It is generally observed that bad hygiene of fields, particularly during the critical stage of crop growth (November–May), was responsible for loss of planting material due to rodent attack. Fresh as well as old burrows are distinctly visible in saffron fields. The extent of damage to saffron corms by rodents ranges from 10 to 50% (Manzar *et al.* 2008). Due to a traditional longer planting cycle, cultivation on raised beds, mixed cropping with almond and poor weed management, rodents have found saffron fields as a breeding ground due to availability of food material during fall months (October to March), when all other agricultural fields are fallow and thus devoid of any grain to be used as a food material by rodents. Manual weeding (pre- as well as post-winter) has been found to be effective in the control of rodents, enhancing growth of saffron plants with a significant effect on improving corm and saffron yield.

Smaller flowers

There are many genetic and environmental factors affecting flower size and ratio of different parts of the flower. Environmental factors such as physical and chemical properties of the soil, time of harvesting the flowers, age of the corms and cultural practices can influence the size of the flower and stigma inside it (Mir 1992; Kafi *et al.* 2006).

In Khorasan, 78.5 kg of fresh flowers (equal to 170,000 flowers) are required for producing 1 kg of dry stigma and style (standard saffron). This means 1 kg standard saffron of Khorasan is collected from 2165 fresh flowers (Mollafilabi 1994; Kafi *et al.* 2006). On the other hand, the number of flowers required per kg of standard saffron of Kashmir varies between 2680 and 3840, indicating that the saffron flowers of Kashmir are smaller than those of Khorasan (Kafi and Showket 2007). In other words, labour costs engaged per unit area per operation will be higher in Kashmir than in Iran.

Poor post-harvest handling

There are data available that point to the diverse quality of saffron with different major traders of Kashmir State, with very few conforming to the norms set by the Indian Standards Institute (ISI 5453) for Indian saffron (data not shown). One major reason for this diversity in quality is poor post-harvest handling and storage. Quality evaluation of Kashmir saffron has confirmed its intrinsic high quality with respect to colouring pigment, and traditional postharvest processing is responsible for its deteriorating quality (Nehvi *et al.* 2005). There is a high percentage of crocin (14-17%) in the fresh stigmas of Kashmiri saffron (Nehvi *et al.* 2007c). However, the practices followed by farmers for harvesting flowers, separating stigmas, drying, packing, storing and marketing bring down the crocin content to 9-11.5%. As such there is a need for awareness programmes for improving postharvest handling, educating farmers about the benefits of picking flowers at the right stage, separating stigmas and style in the shortest possible time, popularizing the use of solar dryers, quality evaluation and branding, etc.

1. Picking and sorting

In Kashmir, flower picking is not usually done daily. It is usually done once every four days, before 9 a.m. This should be done on a daily basis because flowers are short-lived and if they are left for a longer period, not only can they get damaged, but the quality of saffron also decreases (Mir 2002; Munshi *et al.* 2002). Flowers should be carried in clean baskets and should not be overloaded as that may prevent free air circulation.

A delay in flower picking from the date of flower opening and a delay in separation of the stigma from the flower contribute to the drop in crocin content. Flowers picked on the 4th day of emergence give maximum recovery (weight) of stigmas and pistils, called *Laccha* (0.760 g per 100 flowers) and crocin content (12-13%). The sorting of stigmas from stamens and the remaining floral matter is a

crucial stage of the processing. If the separation of stigmas is completed within 6-8 hrs after picking, more than 95% of the saffron is recovered, while 24 hrs after picking only 40-50% is recovered. After 72 hrs of picking, the entire saffron flower becomes a cake-like mass with no recovery (Nehvi *et al.* 2004). During all these operations the processors of their product should always keep in mind the need to keep all surfaces clean. In order to comply with ISO specifications it is a good idea to repeat sorting 2–3 times per batch.

2. Drying

Drying is a critical step in saffron processing. Drying brings about the physical, biochemical and chemical changes necessary for imparting the desired attributes to saffron. Washing saffron to remove foreign matter (dust, mud, parts of insects, etc.) is strictly prohibited because its principle constituents, the crocins, are water-soluble. In addition, like all carotenoids, these are also light sensitive and hence exposure to light throughout processing should be a minimum. Picrocrocin, the bitter constituent, decreases during drying and the subsequent treatment steps, whereas safranal, absent before drying and the period just after that, starts to develop in the first period of storage (Ordoudi and Tsimidou 2004).

Saffron drying depends heavily on temperature and relative humidity of the drying room. In Kashmir, the traditional drying practice followed by farmers takes about 27-53 hrs in shade leaving a moisture level of about 10%, which is higher than the recommended level (8%). Moreover, a longer drying period (27–53 hrs) adversely affects the quality, possibly due to both biodegradation and oxidative destruction of the principle components (Sampathu *et al.* 1984). Raina *et al.* (1996) elaborated some drying schemes at a laboratory scale for samples collected from Kashmir. The drying methods employed were (i) shade drying, 4-18°C; ii) sun drying, 11-h photoperiod per day, 12-21°C; iii) solar drying, highest interior temperature 49°C (29°C higher than ambient; iv) dehumidification drying over Si-gel (blue), 40°C; v) in a vacuum oven at 40, 50 and 65°C and at reduced pressure of 40 mm; vi) in a cross-flow oven at 20, 40 and 50°C and vii) in an electric oven at 40, 50, 65 and 80°C. The optimum tray-load was found to be 1 kg m^{-2}. The proposed ideal temperature for artificial drying was 40 ± 5°C. At lower temperatures, lengthy periods of processing were experienced that resulted in pigment loss whereas at 50 or 60°C thermal degradation of pigments was observed. For example, using electric oven and cross-flow drying methods the crocin content was 154 and 156 g kg^{-1}, respectively at 40°C, while it was much lower (124 and 129 g kg^{-1}) at 20°C and slightly lower (148-150 g kg^{-1}) at 50°C. Vacuum and cross-flow drying caused a significant reduction in safranal content (200-350 g kg^{-1}) but at the same time increased levels of 4-β-hydroxysafranal (500-700 g kg^{-1}), causing it to have unpleasant sensory characteristics. Samples dried in shade or under accelerated conditions had a better flavour profile that coincided with higher amounts of safranal (550-680 g kg^{-1}) and lower levels of 4-β-hydroxysafranal (140-200 g kg^{-1}).

3. Decontamination

Spices may be highly contaminated with moulds, yeasts and bacteria, either as vegetative cells or spores coming from plants, soil, or the faeces of birds, rodents, insects, etc. Contamination may occur during harvesting, handling, transportation or storage of the spices (Sjöberg *et al.* 1991). Considering their high microbial load (10^3–10^8 organisms per g), it is obvious that when they are used untreated they may well cause several food-borne diseases. Fortunately, for public health, herbs and spices are used in minor quantities and risks are, thus, reduced. In Kashmir, no decontamination practices are followed, as decontamination by chemical treatment (ethylene oxide, propylene oxide and methyl bromide) or irradiation leave toxic residues and adversely affect organoleptic properties (Sjöberg *et al.* 1991). The effect of γ-irradiation on the colour and flavour of saffron has indicated a perceptible deterioration in the oil obtained from the samples treated at doses higher than 5 kGy (Zareena *et al*. 2001). A substantial decrease (*ca*. 90%) was noticed in the crocins content of irradiated stigmas of saffron and a concomitant increase in the crocetin level. They suggested a cleavage in the glucosidic linkages that may occur during irradiation causing an about 2-fold increase in the crocetin content corresponding to a 83–89% loss in crocin content. These findings led to the conclusion that irradiation doses for this spice should not exceed 5 kGy and thus decontamination using γ-irradiation can be applied to saffron only when having a low microbial load.

4. Packaging

Dried saffron is vulnerable to moisture ingression, light and air (photo-oxidation). Once exposed to these conditions hydrolysis of crocin into crocetin occurs reducing the quality. Packaging of a dried product is normally done in poly bags by farmers and later (between 1-6 months) sold to wholesale traders. Saffron growers of Kashmir usually store saffron in earthen pots or polythene bags without taking care of moisture (Mir *et al.* 2008). Saffron packed in 10 gauge polythene bags and stored at ambient temperature in the dark over a period of one year loses 60-70% of its crocin content (Mir 2002). Additionally, due to high moisture content on account of traditional drying the saffron loses its colouring strength during storage and becomes inferior.

India should formulate suitable guidelines and methods of harvesting, separation, drying and storage of saffron for Kashmiri saffron farmers, on a similar pattern as in Iran. In Iran Institute of Standard and Industrial Research Organization (ISIROI) has released "Control Points of Harvesting and Processing of Saffron" with the aim to introduce suitable methods of harvesting, separation, transportation, drying and storage of saffron. In addition, in Iran, the Hazard Analysis Critical Control Points (HACCP) states the best way for analyzing hazardous risks and critical control points from the time of flower harvesting to stigma packaging, and is implemented by companies dealing with drying, packing and export of saffron.

Urbanization and pollution

The traditional saffron belt of Pampore is located on the national highway connecting Srinagar (capital city of J& K) to the rest of the country. It is barely 20 km away from the city centre and hence lucrative for development of satellite townships by real estate developers. Due to the high rise in price of the land in Srinagar, the land brokers have been trying hard to divert saffron fields towards construction business.

In addition to the air pollution caused by heavy vehicular traffic in the traditional saffron belt, recently mercury accumulation in saffron fields close to a cement factory has been reported by Jan and Bhat (2008). Increased mining and high rate of fossil fuel burning by the cement factory have been cited as the main sources of mercury pollution.

CONCLUSIONS

In Kashmir, the saffron crop is totally rainfed and if rains are received at the sprouting and pre-flowering stages, flowering is optimum and saffron yields are normal. Any major drop in precipitation at these stages or unseasonal rains during October cause serious damage to flowering and hence saffron yields. The major factors responsible for lower production and productivity in Kashmir can be summarized as: (i) Inadequate moisture management, a critical input to initiate timely growth of roots and floral/aerial vegetative shoots under rainfed conditions on uplands; (ii) Planting of small and defective corms of different grades as opposed to selected corms (> 2.5 cm diameter); (iii) Planting of untreated corms vs the recommended fungicide-

treated corms; (iv) Longer planting cycles of 10-15 years vs annual planting in Italy and a 5-7 year cycle in Spain and Greece, and 6-15 years in Iran; (v) Non-application of organic manures in contrast to the recommended carpet dressing of well decomposed organic manure; (vi) Poor weed management; (vii) Poor post-harvest handling practices and storage; (viii) Inhibition of the adoption of improved production practices and lack of scientific aptitude among small and marginal farmers.

In the last decade Iran has shown a continuous expansion of the saffron-growing area with a mean annual growth rate of 11.5% compared to a 7.5% annual decline in Kashmir. The shorter age of saffron fields, high planting density, proper nutrient management system with adequate facilities of irrigation, non-incidence of corm rot and proper weed and insect management are the key factors for higher saffron production in Iran, all of which are lacking in Kashmir.

With a recent increase in world price of saffron, some farmers have shown interest in improved technologies and adoption of scientific techniques for commercial benefits. Furthermore, there is a need to evaluate the possibility of 'organic farming' of saffron in fields or indoors for improving farmers' income opportunities.

ACKNOWLEDGEMENTS

The first author acknowledges the ideas and inputs received from various saffron growers and traders in India, and highly appreciates the support given by officers of J&K Government.

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Sustainable Saffron (*Crocus sativus* Kashmirianus) Production: Technological and Policy Interventions for Kashmir

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ABSTRACT

The Kashmir valley is well known for quality saffron but since the last decade production and productivity of this crop has shown a declining trend in Kashmir. This paper emphasizes ample scope for maximizing profitability of this crop for Kashmir saffron growers, provided that sincere efforts are made. Initiatives are needed for reversing this declining trend by adopting strict quality control measures, preventing adulteration, mechanizing production and introducing marketing interventions. Adoption of novel scientific technologies, including biotechnology, can go a long way to reduce the costs of saffron production in the future.

Keywords: adulteration, biotechnology, government, mechanization, quality, tissue culture
Abbreviations: BA, 6-benzyl adenine; **BAP**, 6-benzyl amino purine; **2,4-D**, 2,4 dichlorophenoxy acetic acid; **GA_3**, gibberellic acid; **IAA**, indole-3-acetic acid; **Kn**, kinetin; **MS**, Murashige and Skoog; **NAA**, α-naphthalene acetic acid; **TDZ**, thidiazuron

CONTENTS

INTRODUCTION

Saffron (*Crocus sativus* Kashmirianus) covers about 4% of the total cultivated area of the Kashmir valley and provides about 16% of total agricultural income (Anonymous 2008). Saffron is chiefly grown in the following districts: Pulwama (74.64%) comprising Pampore, Balhuma, Wayun, Munpur, Mueej, Konibal, Dus, Zundhur, Letpur, Sombar, Baras, Ladu and Khrew; Badgam (16.13%) comprising Chadura, Nagam, Lasjan, Ompora and Kralpura; Srinagar (6.68%) comprising Zewan, Zawreh and Ganderbal; Doda (2.50%) comprising Poochal, Namil, Cherrad, Huller, Blasia, Gatha, Bandakoota and Sangrambatta, and some areas of Anantnag district comprising Zeripur, Srechan, Kaimouh, Samthan and Buch. Saffron cultivation forms an important sector for the livelihood security of more than 16,000 farm families located in 226 villages. The limited size of land holdings makes cultivation less profitable, with over 61% of holdings

Table 1 Test value range of Kashmir saffron as per the procedure of BIS (IS 5453 Part 2): 1996, ISO-3632-2: 1993.

Parameters	Total value range
Isolated stigmas (%m/m)	0.00-71.15
Un-isolated stigmas (%m/m)	15.75-96.76
Floral wastes (%m/m)	3.04-21.75
Extraneous matter (%m/m)	0.05-0.58
Moisture and volatile matter (%m/m)	9.78-13.69
Bitterness (as direct reading of absorbance at 257 nm)	42.00-75.00
Saffranal (as direct reading of absorbance at 330 nm)	27.00-41.00
Colouring strength (as direct reading of absorbance at 440 nm)	97.00-172.00
Total ash (%m/m)	4.60-4.95
Acid insoluble ash (%m/m)	0.86-1.61
Total nitrogen (%m/m)	2.06-2.68

below 0.5 ha, and only 26% of holdings between 0.5-1.0 ha and 13% of holdings > 1.0 ha (Anonymous 2009). The total area under this crop in the State of Jammu & Kashmir has shown a decrease of 83% in the last decade, a 215% decrease in production and a 72% decrease in productivity (Husaini *et al*. 2010).

In the present paper we highlight the major causes for diminished export potential, and also suggest some technological and policy measures for sustainability of saffron production and revival of the saffron industry.

SAFFRON TRADE

Until the beginning of the 1980s, Spain almost monopolised the world saffron trade (>90%). Its production accounted for 52% of overall global saffron production while India (Kashmir), Greece, Italy and France produced 21.2, 13.2, 7.5 and 6.1%, respectively (Sampathu *et al.* 1984). At the end of the 1990s, significant changes in production trends were observed, with Iran taking a lead by annually producing 80 tons (t) of saffron, followed by Kashmir (10 t), Greece (6 t), Spain (3 t) and Morocco (1 t) (Saltron *et al.* 1999; Alonso *et al.* 2001). Minor quantities of saffron are produced in different countries throughout the globe, viz. China, Afghanistan, France, Switzerland, Turkey, Azerbaijan, Japan, Australia (Tasmania), New Zealand, Argentina, and the USA (de los Mozos Pascual *et al*. 2010).

Kashmir saffron is exported mainly to Spain, France, USA, UK, UAE, Israel, Japan, etc. The exports have declined by about 87% from 9.77 tonnes in 1998-99 to 1.30 tonnes in 2005-06, due to the declining trend in domestic and international prices during this period (Anonymous 2007). Recently, increasing costs of saffron producers in the EU can open avenues for Kashmir to offer saffron to the world market in larger quantities (Fernández 2007). However, aggressive exports from Iran, with 90% of produce being exported, have challenged saffron exports from India (primarily Kashmir).

SAFFRON QUALITY

According to the definition given by the Food and Agricultural Organisation (FAO), saffron forms "a loosely matted mass of dark, reddish-brown flattened threads, amongst which a few narrower yellow ones can be distinguished. The upper, enlarged part of the flattened threads is the stigma of the flower, the lower narrower portion is the style" (FAO 1986).

The quality and consequently the commercial value of saffron are based on an estimation of colouring power, bitter taste and aroma. The quality of saffron is certified in the international trade market following the International Organisation for Standardisation (ISO) 3632 Normative since 1993. The ISO issued a specific standard for saffron ISO 3632 in 1975, revised it in 1980 and technically improved it in 1993. In ISO 3632 1&2 (1993) trade standard definitions as well as requirements for saffron quality and methods of analysis are given as follows: (i) Saffron in filaments are the stigmas of *Crocus sativus* Linneaus, dried, dark red in colour and trumpet shaped, serrated or indented at the distal end. The length is between 20 and 40 mm. The stigmas may be isolated or joined in pairs or threes at the end of the portion of the style, which is white/yellow in colour; (ii) Saffron in cut filaments are the stigmas of *C. sativus* with styles removed and completely detached from each other; (iii) Colouring strength is mainly due to its crocin content, as measured by its optical density at about 440 nm; (iv); Bitterness is mainly due to its picrocrocin content, as measured by its optical density at about 257 nm; (v) Flavour is mainly due to its safranal content, as measured by its optical density at about 330 nm.

In Iran the agency responsible for guarding the quality standards of products, including saffron, is the Institute of Standard and Industrial Research Organization (ISIROI). Its main aim is to specify the norms of packing, labeling and sampling, and methods of testing (for filament and powder form). In 1993, the organization introduced and published guidelines for saffron standards under the title "Saffron Specifications". There is another part of the standard (Saffron Test Method) that introduces methods for testing saffron and is applicable to filament and saffron powder. It explains general tests such as moisture and volatile matter, colour strength, bitterness, flavour, floral waste content, microscopic examination of saffron powder, determination of total ash and acid-insoluble ash together with measurement of crocin, picro-crocin and safranal by spectrophotometry. Besides, there is one more standard (Microbial Specification and its Tests) that examines microbial contamination during picking of flowers, flower transportation, stigma separation, drying and packing.

In India, the agency that sets up and guards the quality standards of saffron products is the Bureau of Indian Standards (BIS), and 'saffron specification' is defined in IS5453: Part 1 (1996), which is equivalent to ISO3632-1 of 1993, and 'saffron methods of test' are defined in IS5453: Part 1 (1996), which is identical to ISO3632-2 of 1993. Following the saffron test method as defined in IS5453: Part 1 (1996), various samples of saffron from Kashmir have been analyzed, which indicated a wide range of test values for various parameters (**Table 1**) (Anonymous 2008).

The chemical composition of dried saffron stigmas has been extensively studied since the end of the 19th century. Proximate composition of dried stigmas of saffron indicate that they contain water (10–12%), mineral matter (5–7%), fat (5–8%), protein (12–13%), reducing sugars (20%), free sugars (trace), starch (6–7%), pentosans (6–7%), gums and dextrins (9–10%), crude fibre (4–5%), crocin pigment (8–9%) and essential oil (0.3%) (Sampathu *et al.* 1984; Ríos *et al.* 1996).

Crocin

Saffron colouring properties are mainly attributed to the water-soluble crocins. Pfander and Rychener (1982) found that crocin 1 represents 40–45% of the saffron aqueous extract, followed by the crocetin-(β-D-gentiobiosyl)-(β-D-glucosyl) ester (35%), the crocetin-di-(β-D-glucosyl) ester (10%), as well as the crocetin-mono-(β-D-gentiobiosyl) and

mono-(β-D-glucosyl) esters (each 2%). Alonso *et al.* (2001) examined the content of Spanish, Indian and Iranian saffron in crocin derivatives and gave results for *trans*- and *cis*-crocins (*trans*-crocin: 0,46–12,12%; *cis*-crocin: 0,04–8,53%; *trans*-(β-D-gentiobiosyl)-(β-D-glucosyl) ester: 0,01–9,44%; *cis*-(β-D-gentiobiosyl)-(β-D-glucosyl) ester: 0.01–2.26%). Similarly, Caballero-Ortega *et al*. (2004) quantified the contents of *cis*/*trans*-crocins from saffron samples of Azerbaijan, Kashmir, Iran and Spain and found that the total carotenoid content in Azerbaijan and Iran was higher than other samples. This difference was attributed to the geographical location or the degree of purity of these samples.

Picrocrocin

The colourless glycoside picrocrocin ($C_{16}H_{26}O_7$, 4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde) is the major bitter compound of saffron. Alonso *et al.* (2001), who examined several samples of Spanish, Indian and Iranian saffron, found differences in picrocrocin content: 0.79–12.94% in Spanish saffron, 1.07–2.16% in Indian saffron and 2.18–6.15% in Iranian saffron. Caballero-Ortega *et al*. (2004) quantified the contents of picrocrocin from saffron samples of Azerbaijan (2.69%), Kashmir (0.39%), Iran (3.24%) and Spain (1.02%).

Safranal

Safranal is the main compound responsible for the aroma of saffron, even though there are other more volatile constituents which provide saffron with its final odour (Zarghami and Heinz 1971; Sampathu *et al.* 1984; Curro *et al.* 1986; Tarantilis and Polissiou 1997; Straubinger *et al.* 1998). The levels of safranal and of the other aroma compounds of saffron vary depending mainly on the conditions of processing and storage, and on the methods of analysis. Safranal content of saffron samples from Azerbaijan (0.859%), Kashmir (0.552%), Iran (0.281%) and Spain (0.582%) showed significant differences between samples (Caballero-Ortega *et al*. 2004).

Crocin, picrocrocin, safranal standardization

The above results clearly show that the levels of each of these important biomolecules (crocin, picrocrocin, safranal) vary depending on the origin and overall processing conditions and storage length. The above results point towards comparatively poor quality of Kashmir saffron. On the contrary, in a study different approaches, including colourimetry (Hunter Lab), UV-visible spectroscopy (ISO 3632-2003) and APCI-MS (atmospheric pressure chemical ionisation-mass spectroscopy), were employed for comparing saffron samples (in filament forms without adulteration) from the UK, Iran (Birjand) and India (Kashmir) in terms of three main components of saffron (colour, odour and taste) (Yadollahi *et al.* 2007). Colouring strength (crocin) and aromatic strength (safranal) values of saffron solutions from the three countries were ranked as India > Iran > UK, while the mean bitterness value of Iranian saffron was greater than that of Indian saffron followed by UK saffron. Yadollahi *et al.* (2007) characterised saffron samples uniformly based on the method specified in ISO/TS 3632-1 and 2, and later compared them with values obtained from ISO/TS 332-1 as chemical requirements for saffron samples. The methodology used in the study further points out that care must be taken when comparing samples, i.e., a comparison must be made between equivalent samples (of the same quality grade) and saffron samples must be bought directly from the producers. Nevertheless, the reasons for these differences need to be investigated in greater detail to determine if this is due to inherent poor saffron quality based on geographical origin or due to impurities/adulteration or poor post harvest handling and storage.

ADULTERATION

Saffron is considered to be the highest priced spice in the world (on average, 500 $/kg). Its high value makes saffron the object of frequent adulteration and fraud (Fernández 2007). Oberdieck (1991) and Alonso *et al.* (1998) summarised the most frequent adulteration practices: (i) Misbranding or origin falsification; (ii) Admixture with old saffron or with style material; (iii) Admixture with stamens previously cut and dyed; (iv) Impregnation with substances to increase weight (syrups, honey, glycerin, oils, potassium hydroxide, saltpetre, Glauber's salt, Seignette's salt, borax, lactose, starch or glucose); (v) Parts of other plants with or without colouring power; (vi) Animal substances (fibers of salted and dried meat); (vii) Threads of coloured gelatin; organic colourings and colouring materials derived from tar.

Adulteration in saffron is rampant and a serious malpractice in Kashmir. Imported Iranian saffron is mixed with local saffron and sold as 'Kashmir brand' at a higher price. The exact figures of saffron routed into Kashmir are not known as most of the imports are clandestine in nature. In addition, ray florets of African marigold, sugar-coated paper cuttings, dried and meshed flesh fibres, dyed saffron stamens, dyed stigma of maize (corn silk), etc. are also mixed by corrupt traders. Fats, oils and glycerine are sometimes used to increase the weight of saffron. This adulterated and spurious saffron is sold to ignorant tourists who visit the Kashmir valley in large numbers.

According to a study on saffron sold in Kashmir, only 52% is genuine, 30% is poor grade and 17% totally adulterated (Mir 2002). Saffron produced in the Kashmir valley is classified as 'Special' (also called Mongra) when it contains only dried stigmas with deep red colour, ≤ 5% floral waste and ≤ 0.5% foreign matter (**Fig. 1A**). It is classified as 'Standard' grade (also called Laccha) when it contains stigmas mixed with styles, is of light red colour, ≤ 10% floral waste and ≤ 1.0% foreign matter. The saffron produced in Doda district of Jammu Province is equivalent to Laccha of Kashmir valley and is locally known as 'Guchhi' (Dhar and Mir 1997).

Microscopic observation of typical anatomical elements is valuable to test the authenticity of powdered saffron. Among the diagnostic characters are the upper epidermis of the stigmas with small papillose protuberances and large pollen grains (Ordoudi and Tsimidou 2004). Determination of the major organoleptic characteristics of saffron (picrocrocin, safranal and crocins) is carried out in an oversimplified way by spectrometric evaluation of an aqueous extract at three characteristic maxima (257, 330 and 440 nm, respectively). Molecular tools may be ideal for checking purity of product, even after processing, acting as markers for adulteration (with other plant species) (Khan *et al*. 2008).

Lack of laboratories to evaluate quality coupled with ineffective law enforcement are the major constraints in enforcing adoption of quality standards and uniform price fixation as per the standards in Kashmir. The Food and Adulteration Act of India (1949) is the only major law which the State agencies use to catch adulterators to save the saffron industry from this menace, although how many have been prosecuted is unclear.

DISORGANIZED MARKETS AND LACK OF PROPER FINANCING

An economic analysis of costs and returns, net present value, benefit cost ratio, pay back period, internal rate of return and the farm profit measures indicate that saffron is economically viable for cultivation under Kashmir conditions (Anonymous 2004; Wani *et al.* 2008). The economic analysis of saffron in Kashmir (District Pulwama) revealed that benefit cost ratios (BCR) were 2.31 and 2.21 at 10 and 12% rates of interest, respectively. This indicates that at the prevailing rate of interest, an investment of $1 fetched a return of $2.31 and $2.21 at 10 and 12% discounting rates, respectively. The undiscounted average BCR was calculated

as 2.97. Since BCR was >1, it showed that the investment in saffron cultivation was economically viable (Wani *et al.* 2008). However, since saffron markets in Kashmir are highly disorganized, a large cut of the profits is taken by private brokers and a long chain of middlemen linking the growers with the consumers. The majority of growers (70.86%) sell their produce through saffron brokers (*dalals*), accounting for 59.67% of saffron production while 16% of saffron growers sell saffron through sub-firms, and only 13% sell it through wholesalers/firms, retailers, government agencies and directly to consumers (Wani *et al.* 2008). In an earlier survey Munshi (2002) reported that 70% of saffron growers sell their produce to *dalals* and 25.7% to sub-firms, while only 1.43% sell it directly to wholesalers in the rest of the country (Delhi, Amritsar, Mumbai, Kolkata) and 1% to cooperative societies.

Sometimes brokers spread misinformation through print media about the possibility of a bumper crop in the valley, which causes the market prices of saffron to crash and hence buy saffron at much lower prices from farmers (Nehvi *et al*. 2008). This has discouraged farmers, and hence a strategy needs to be evolved for ensuring maximum returns to the farmers so that it enables them to make additional investments in adopting improved technologies for enhancing saffron production and productivity. There is an urgent need for setting up an organized saffron market centre (locally called 'Mandi') in the traditional area of saffron production, i.e. Pampore.

Until recently, there were no formal channels of financing saffron growers in the state. While banking on an informal financing system, growers had to pay exorbitant rates of interest resulting in thin margins for them and hence small growers showed a lack of interest in saffron cultivation. While appreciating these financial hardships of saffron growers, the J&K Bank (a state nationalized bank) designed a special scheme namely "JK Bank Zafran Finance" exclusively for saffron growers in 2007-2008. All saffron growers, including small, marginal and large farmers including contract farmers engaged in cultivation of saffron or intending to commence cultivation are eligible to obtain a loan under the scheme. The loan covers the entire plantation and production costs, including plant material, agricultural machinery, labour, post-harvest handling and packaging.

INITIATIVES FOR SUSTAINABLE SAFFRON PRODUCTION AND REVIVAL OF THE SAFFRON INDUSTRY IN KASHMIR

The major initiatives that are needed for sustainable saffron production and revival of the saffron industry are discussed broadly next.

Breeding and biotechnology

Saffron is a triploid species with 3n=24, x=8 chromosomes. Its triploid nature allows for vegetative multiplication, but not regular sexual reproduction. This is because meiosis and gamete development in triploids are irregular, resulting in many anomalies in sporogenesis and gametophyte development. Manipulating seed to produce better plants has not been successful in cultivated saffron as meiotic abnormalities result in abnormal chromosome assortment and formation of an abnormal number of genetically imbalanced spores which vary in shape and size, leading to complete sterility (Chichiriccò 1990). Moreover, corm multiplication does not induce genome variations with the exception of some mutations, which are not easily detectable in a triploid saffron population.

1. Collection and conservation of genetic resources

Recently a consortium, composed of 14 groups of 9 EU and non-EU countries has taken the responsibility of creating and maintaining the genetic variability of saffron. The European Commission has approved a project on "Genetic Resources of Saffron and Allies (*Crocus* spp.): CROCUSBANK" to create, characterise and exploit a germplasm collection (bank) in *Crocus* species (Fernández 2007).

There is a need to establish a germplasm bank of saffron in India (preferably in the state of J&K) for collection and reproduction of saffron bulbs from all the areas that cultivate saffron in India. This plant material can then be used in selection programmes all over the country and serve as sources of resistance and other agronomically interesting traits to be transferred between saffron clones through appropriate breeding programmes and technological tools. These objectives can be achieved by a four-pronged strategy: (i) The collection of *Crocus* material by means of requests to different regional centres growing the plants and visiting specific locations at appropriate dates to collect both cultivated saffron species and sub-species; (ii) Multiplication of collected plant material for conservation in the Plant Germplasm Bank using tissue culture techniques; (iii) Preparation of a list of descriptors for primary characterisation of the collected material; (iv) Providing material to potential users by distribution of corms, tissue culture and DNA samples.

2. Primary and secondary characterization of genetic resources

Primary characterization of the collected material based on the phenotypic characters with good heritability i.e. morphological (floral features, corm size), cytological (chromosome numbers, genome size and ploidy level), phytochemical (saffron chemical composition) and molecular (DNA analysis) studies are necessary. In view of stresses due to climate change, there is an urgent need to identify genotypes from marginal areas characterised by soil salinity and water stress. The collection of *Crocus* species needs to be evaluated in different stress levels for the number of flowers harvested per plant, fresh and dry style weight and chemical compounds of each sample.

The intensive cultivation and mono-culture of saffron in saffron-growing belts of the Kashmir valley together with the continual use of diseased material results in the frequent occurrence of corm rot diseases caused by different pathogens (Madan *et al.* 1967; Dhar 1992; Thakur *et al.* 1992; Wani 2004; Ahmad and Sagar 2006; Kalha *et al.* 2007; Husaini *et al*. 2010).

Screening *Crocus* accessions against these saffron pathogens is required to find genetic resistance. The number of infected corms in each accession can be determined by the presence of brown to dark brown sunken and irregular patches below the corm scales (Ghani *et al.* 2002).

3. Selection

The genetic base of natural saffron populations around the world is very narrow and no significant improvement in productivity is expected through recurrent selection (Brandizzi and Grilli-Caiola 1996). Still, it would be worthwhile to continuously select well developed corms from a population for improved economically important characteristics like long red stigmas (Grilli-Caiola 1999). This method offers an advantage in maintaining the genetic characteristics of the plant, but it does not allow for making any genetic improvement.

In Kashmir, the cultivated population of saffron is *Crocus sativus* 'Kashmirianus', which is recognized by its extremely dark maroon-purple hue, and is among the world's darkest, suggesting strong flavour and colourative effects. Surveys undertaken to study the extent of variation revealed a wide spectrum of variability in saffron flowers and corm samples collected from saffron-growing areas of Kashmir (Dhar *et al.* 1998). Stigma length varied from 1.75-3.72 cm and style length ranged from 1.70-4.25 cm. The average number of daughter corms per mother corm ranged from 2.37-7.05 and their average weight ranged from 1.59-8.49 g. Crocin content ranged from 8.55-17.10%. Latto and Dhar

(1999) studied temporal saffron populations of Kashmir for 6 floral characters viz., number of flowers per spathe, fresh flower weight, flower size (perianth area), stigma length, fresh stigma weight and dry stigma weight. Appreciable differences in coefficient of variation for all the characters were observed. Maximum coefficient of variation was recorded for flowers per spathe (59.15), whereas minimum (0.42) was recorded for stigma length. Similar results of a wide range of variability in Kashmir have also been reported by Zargar (2002). Stigma length ranged from 2.41-3.87 cm, and crocin content ranged from 9.92-14.35%. Based on the reports of the extent of variability in natural subpopulations of saffron in Kashmir, the identification of elite genotypes is imperative for the Kashmir saffron industry. Collection and evaluation of saffron germplasm in Kashmir has led to the identification of 10 elite clones with distinct yield superiority (SMD-3, SMD-11, SMD-31, SMD-45, SMD-52, SMD-68, SMD-79, SMD-81, SMD-21 and SMD-224). The saffron yield of elite genotypes ranged from 4.0-7.6 kg/ha with a corresponding crocin content ranging from 13.89-17.10% (Nehvi *et al.* 2007a). Identification of these 10 clones with distinct yield superiority of 50-170% above the average of a natural heterogenous population is a milestone for the Kashmir saffron industry. Presently the area cultivated with heterogenous populations is 3110 ha (2007-08) with average productivity of 1.62 kg ha^{-1}. Replacing the heterogenous population with an elite saffron clone, e.g., SMD-45 with an average productivity of 7.6 kg ha^{-1}, would increase saffron production to 23.63 tonnes, a 366% increase. The yield superiority of this clone is attributed to by Nehvi *et al.* (2007a) to its superior traits: more flowers/ spathe, fresh pistil weight and increased stigma and style length.

Therefore, utilization of heterogeneity in the natural population and development of high-yielding genotypes using the existing gene pool offers opportunities for improving the productivity of this crop.

4. Creation of genetic variability

Saffron mutagenesis is a useful method for increasing genetic variability, which can be later exploited through selection. Mutagenesis in saffron should be done when the floral shoot emerges from the corm, as meristem differentiation and maximum mitotic activity takes place during this stage and chimera formation is low (Laneri 1990). Akhund-Zade and Muzaferova (1975) irradiated saffron corms with gamma rays, which resulted in an increase in corm production, flower number and stigma weight. Contrastingly, Laneri *et al.* (1990) observed only morphological variants and no useful mutant could be recovered. There have been very limited efforts by Indian researchers to increase genetic variability in saffron using non-conventional breeding techniques, such as irradiation for inducing mutagenesis and colchinisation for inducing polyploidy (Zaffer *et al.* 2004; Nehvi *et al.* 2007a). A comparison of morphological characteristics (number, shape, size and texture of organs) in triploid and probable colchiploid variants of saffron revealed that the variant plants exhibited a slower rate of growth than triploids in vegetative and flowering stages (Zaffer *et al.* 2004). Nehvi *et al.* (2007a) irradiated saffron corms with gamma rays from a Co^{60} source at 0.25, 0.50, 0.75 and 1 Kr doses. M1 mutants with 0.25 Kr showed 53.44 and 84.28% enhanced corm yield and saffron yield, respectively, while at 1.0 Kr plant survival was reduced to 79% (Nehvi *et al.* 2007a).

Inter-specific hybridization of saffron is another area that needs attention as it can generate variability for breeding. Introgression of genetic traits from closely related wild species into the saffron genome can result in resistance to pathogenic fungi and viruses (Russo *et al.* 1979), and improvement of the productivity and quality of saffron drugs (Negbi *et al.* 1989).

The pistils of saffron are receptive to pollen from other *Crocus* spp. viz. *C. thomasii*, *C. hadriaticus* and *C. oreocreticus* (Chichiriccò 1989). The hybrid seeds with *C. thomasii* as pollen parent germinate and produce plants, while interspecific crosses with *C. sativus* as pollen parent fail to germinate (Chichiriccò and Grilli-Caiola 1984, 1986; Chichiriccò 1989). This failure can be overcome by *in vitro* culture of cross-fertilized saffron ovaries and successful seed set may be accomplished.

5. Tissue culture

Reproduction in saffron is vegetative, dependent on humans and slow (Wani and Mohiddin 2009). Conventional methods are insufficient; therefore, saffron cultivation needs efficient mass production of pathogen-free corms. Micropropagation of saffron has therefore been advocated as the best alternative for its propagation (George *et al.* 1992; Ahuja *et al.* 1994). Tissue culture has been used for many genotypes, including saffron (Bagheri and Vasel 2006). During the past 30 years a number of attempts have been made by different workers across the globe having different objectives for *in vitro* studies in saffron, which are reviewed next.

***In vitro* corm production:** *In vitro* technology to increase the reproductive capacity of saffron will go a long way to overcome the low corm multiplication ratio (Dalal 2002). The earliest record of tissue culture of *C. sativus* is by Paradics (1957), who reported leaf and corm production from explants pretreated with ethylene. Later on, after a long gap, mini corm-like structures developed from corm callus which subsequently proliferated and even germinated (Homes *et al*. 1987). Corms can be generated from a variety of saffron explants producing regenerative calli even after a series of subcultures over several years (Homes *et al.* 1987). Cormlet and seedling formation has also been reported by Gui *et al*. (1988) in explants excised from dormant corms. Microsurgery of apical buds combined with ethylene pre-treatments (1000 mgl^{-1}) can increase corm production (four corms developed at the base of each apical bud where other sprouting buds developed into mini corms) substantially (Plessner *et al.* 1990). Dhar and Sapru (1993) then also succeeded in *in vitro* production of corms and shoot-like structures from callus on MS medium augmented with kinetin (Kn) and α-naphthalene acetic acid (NAA). Continued efforts in this direction lead Aguero and Tizio (1994) to obtain *in vitro* mass corm production from branches raised *in vitro* from culturing lateral buds of corms. On the other hand, corms were also induced on shoot explants and callus occurred optimally at 10°C (Milyaeva *et al.* 1995). A few years later microcorm production was again noted by Ebrahimzadeh and Rajabian (1998) and Piqueras *et al*. (1999). Organogenesis via callus formation from bulblets and its consistent production of corms/cormels has been standardized and rooting obtained in 50% of plantlets (Anonymous 1999, 2000). However, field trials in traditional areas of cultivation have not been conducted, which is a major constraint.

In recent years, more information regarding corm production has been published from diverse quarters. An important study on the comparative effect of 6-benzyl amino purine (BAP) and thidiazuron (TDZ) on multiplication and induction of independent micro-corms was carried out by Blazquez *et al*. (2001). The results of that study showed that 0.1 mg/l TDZ with 60% regenerted significantly more fully developed leaf primordia than 2 mg/l BAP, which resulted in only 20% regenerants. That study further revealed that TDZ could accelerate the recovery of complete development of plantlets for rooting and *ex-vitro* acclimatization. More recently, corm segments with buds were cultured on Murashige and Skoog (MS) media containing 2 mg/l BAP and 0.5 mg/l NAA, which favoured the production of 2-3 cormlets/explant. The developing corms were later transferred to pots for greenhouse growth (Karaoğlu *et al.* 2007). Concurrently, *in vitro* mini-corm production has also been obtained by culturing basal leaf segments of saffron on MS medium containing 4.0 mg/l BA (6-benzyl adenine, same as BAP) and 0.5 mg/l NAA (Raja *et al.* 2007). Sucrose plays a

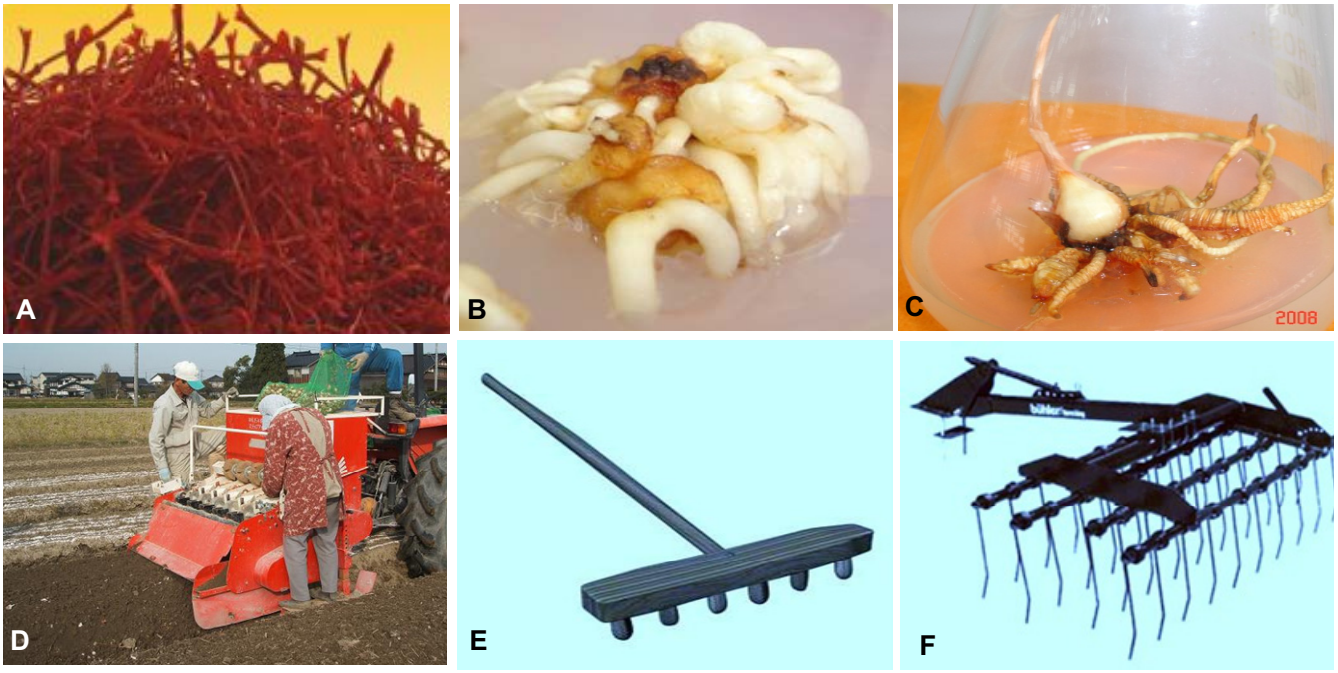

Fig. 1 ***In vitro*** **regeneration of saffron and its mechanization.** (**A**) Special '*Mongra*' saffron of Kashmir. (**B**) Multiple cormlet production *in vitro*. (**C**) Increase in cormlet size and multiple thick root formation. (**D**) Tulip planter. (**E**) Locally made raker, used for hoeing. (**F**) Company-manufactured harrow.

vital role in cormlet production in saffron as an increase in sucrose concentration results in an increase in the osmotic pressure, inhibiting the vacuolation and shrinkage of cytoplasm in cells and thereby increasing the amount of biomass accumulation (Sharma *et al.* 2008). Similar observations have also been recorded where 15 µM BAP with 30 g/l sucrose was not as effective as 15 µM BAP with 70 g/l sucrose as far as cormlet number was concerned; among various trials carried out, 26.4 µM BAP with 30 g/l sucrose favoured multiple mini-corm production most from sub-cultured callus raised from corm slices (Quadri *et al.* 2008). This shows that growth additives also have an important role to play in cormlet induction. In another experiment, Quadri *et al*. (2010) noted that 20 µM BAP and 20 µM NAA most effectively induced higher cormlet number (mean = 20) from corm slices indirectly through callus (**Fig. 1B**). The same group also studied the potential of *in vitro* raised mini-cormlets and vegetative buds (dormant and active) to increase the size/weight using different plant growth regulators and carbon source. The maximum increase in weight range (1-1.5 g) of mini cormlets was registered with 2 µM BAP, 2 µM NAA and 60 g/l sucrose. In vegetative buds only apical buds from active corms increased in size; this was significantly affected by the carbon source. The maximum increase in mass (1.5-2.0 g) occurred with 2 µM BAP, 2 µM NAA and 40 g/l sucrose; 8.8 µM indole-3-butyric acid (IBA) and 40 g/l edible sugar; 2 µM BAP, 2 µM NAA, 2.5 g/l KCl, 30 g/l sucrose, 40 g/l edible sugar (**Fig. 1C**) (Quadri *et al.* 2010).

Bud differentiation and shoot regeneration: Ding *et al*. (1979) carried out tissue culture studies and reported shoot formation from corm buds. They could also successfully induce callus and subsequent shoot differentiation which lead to plantlet formation when the culture medium was enriched with IAA and 2,4-dichlorophenoxy acetic acid (2,4-D) at 1 mg/l each (Ding *et al.* 1981). Bud differentiation in saffron was reported on MS medium when augmented with 2 µM BAP and 9 µM 2,4-D by Huang (1987) in callus cultures obtained from leaf bases. Using similar medium supplemented with 9 µM 2,4-D, Ilahi *et al*. (1987) reported callus formation from corm explants that showed the potential for bud differentiation. For the development of an efficient regeneration system in saffron, callus was induced on MS medium fortified with 0.5 mg/l 2,4-D and 0.3 mg/l Zeatin (Zea) which was sub-cultured in the presence of 4 µM NAA and 5 µM BA for shoot regeneration (Isa and Ogasawara 1988). Separately, Isa *et al*. (1990) cultured protoplasts isolated indirectly from saffron bulbs via callusing immobilized in Ca-alginate beads on MS agar medium supplemented with 0.1 mg/l NAA and 1.0 mg/l BAP. After 2-3 months shoots and roots regenerated form the callus tissue. Shoot development from corm explants was also promoted by 14-56 µM kinetin Kn or Zea or4.5 µM 2,4-D (Plessner *et al.* 1990). Meristem tip culture and plant regeneration from cultured tissue has been reported to be the only way for the wide production of pathogen-free saffron (Hussey 1975; Debergh and Read 1991) but meristems have frequently been used by scientists for somatic embryogenesis rather than for direct plant regeneration. Aguero and Tizio (1994) noticed bud growth and branch proliferation exclusively from lateral buds of corms when used as explants using MS medium supplemented with 6 µM Zea, 10 µM BAP plus 40 g/l sucrose.

To examine the efficient cultural conditions for shoot regeneration, studies were conducted using calli induced from young ovaries; NH_4^+ inhibited shoot regeneration. Moreover, maximum regeneration frequency was 50% on medium supplemented with 0.5 µM NAA and 0.5 µM BAP (Igarashi and Yuasa 1994). Ovary explants used in some other trials could induce shoots directly with 53.7 µM NAA and 4.44 µM BA and subsequently normal plantlet formation from corms was possible (Bhagyalakhshmi 1999). Ovary explants are only competent to initiate organogenesis, particularly direct shoot regeneration, when at a specific stage of ovary development while 20°C seems ideal for caulogenesis from ovary explants (Bhagyalakhshmi 1999).

Much akin to the work of Isa *et al*. (1990), shoot regeneration was also reported from saffron protoplasts immobilized in Ca-alginate beads but using a 0.5 mg/l 2,4-D and 0.5 mg/l BAP combination instead of 0.1 mg/l NAA and 1.0 mg/l BAP. Shoot regeneration was reported 6-7 months after protoplast isolation (Ebrahimzadeh *et al.* 2000a). More recently, Majourhat *et al*. (2007) reported enhanced plant regeneration from cultured meristems in sprouting buds of saffron corms using MS medium enriched with 5 mg/l BAP. By using this type of explant to initiate axillary shoot cultures, a new micropropagation procedure was developed suitable for clonal propagation or *ex situ* germplasm conservation of selected genotypes. Multiple

shoots were also induced from callus initiated from corm slices on 26.4 µM BAP with a higher sucrose concentration i.e. 50 g/l (Quadri *et al.* 2008) while Sharma *et al.* (2008) found that 80 g/l sucrose was effective for cormlet production.

Somatic embryogenesis: Somatic embryogenesis in saffron has been reported as early as 1992 by George *et al*. (1992), who initiated callus from merismatic regions of corms on MS medium supplemented with 2 mg/l 2,4-D and 0.5 mg/l Kn then subcultured explants in the presence of 2 mg/l IAA, 2 mg/l Kn and 100 mg/l ascorbic acid, which resulted in globular embryo formation and subsequent plantlet formation. Ahuja *et al*. 1994 successfully induced multiple somatic embryos from shoot meristems on MS medium supplemented with 20 µM NAA and 20 µM BAP, which subsequently resulted in plantlet regeneration. Somatic embryogenesis was initiated from shoot meristems on Linsmeier and Skoog (Linsmeier and Skoog 1965) medium containing 5 µM BAP and 5 µM NAA where asynchronous development of somatic embryos occurred; these ultimately germinated and formed complete plantlets (Ebrahimzadeh *et al*. 2000b). Shoot meristems have also been used for inducing embryogenic callus with 4 mg/l Kn and 1 mg/l 2,4-D which was subsequently used for protoplast culture (Karamian and Ebrahimzadeh 2001). Embryogenic callus was frequently induced by Darvishi *et al*. (2007) when using 4.5 µM 2,4-D and 4.4 µM BA. Somatic embryogenesis was initiated in four species of *Crocus*, including *C. sativus* on LS medium fortified with 21.5 µM NAA, 17.8 µM BA or 4.5 µM 2,4-D and 18.6 µM Kn where matured embryos germinated later on; complete plantlets were obtained (Karamian 2004). Regeneration of somatic embryos from callus was improved by enriching the medium with 2.4 µM of jasmonic acid (Blazquez *et al*. 2004b). In their study, Blazquez *et al*. (2004a) indicated an important correlation between the stage of somatic embryogenesis and the type of occurrence and expression of anti oxidant enzymes (superoxidase dismutases and catalase) which could act as markers of embryogenesis. Apart from shoot meristems used as explants for somatic embryogenesis, leaf segments have also been used for raising somatic embryos indirectly from embryogenic callus (Raja *et al*. 2007). Amongst various media tested, MS medium enriched with 10 µM BA and 0.5 µM 2,4-D was quite responsive and embryos germinated after maturation and formed palntlets (Raja *et al*. 2007). Even TDZ, a compound having cytokinin-like activity, has been found to be significantly effective in the induction of somatic embryogenesis from 5 different types of corm explants (terminal or auxiliary buds, upper or lower parts of corm tissue and terminal buds from pretreated corms at 40°C for 2 weeks) and different types of explants showed no significant effect; however, 0.5 mg/l TDZ was the most effective treatment (Sheibani *et al*. 2007); matured embryos thus recovered developed microcorms from their basal part after 3 months. Rajabpoor *et al*. (2007) also successfully achieved somatic embryogenesis from upper and lower corm explants using 20 mg/l 2,4-D and 1.0 mg/l BAP were most effective; lower corm explants were much more responsive, indicated by the number and percentage of embryo response, about 33.3 and 93.3% for the upper and lower part of corm tissue, respectively. Even mature floral bases have also been used for inducing somatic embryogenesis on MS medium containing 2 mg/l BAP and 0.5 mg/l NAA, which ultimately germinated and resulted in cormlet formation (Karaoğlue *et al*. 2007).

Stigma proliferation and synthesis of major secondary metabolites: In saffron, apart from micropropagation studies, attempts have also been made to artificially produce saffron and its active principles through *in vitro* multiplication of stigmas. To this end, Himeno and Sano (1987) cultured young half ovaries excised from young flower buds on LS and Nitsch (Nitsch and Nitsch 1969) media supplemented with 10 mg/l NAA + 1 mg/l Kn and 1 mg/l NAA + 1 mg/l BA, respectively. After 10 weeks, stigma-like structures (SLSs) were formed directly on the explants from which 7.57 µg crocin and 1.19 µg picrocrocin were detected on a dry weight basis. Safranal (0.78 µg) appeared only after heat treatment at 50°C for 120 min. Most importantly, the content of these major secondary metabolites in these SLSs were similar to those in intact young stigmas; they were also morphologically similar (Himeno and Sano 1987). In a separate study, young excised single stigmas or half ovaries were cultured to form SLSs to explore the possibility for industrial production of saffron spice (Sano and Himeno 1987). The maximum number of SLSs was 75/half ovary. Although morphologically these structures were similar and *in vivo* resembled grown intact stigmas, the total length was 80% shorter (1/5 of the size) than *in vivo* grown intact stigmas. Since the frequency of SLSs is still low for industrial production of saffron spice, the observations made can be useful as baseline data to conduct further studies (Sano and Himeno 1987). Apart from using ovaries, SLSs were also successfully produced from flower and petal explants (Namera *et al.* 1987), and from half ovaries (Himeno *et al.* 1988; Kohda *et al.* 1993). Young stigma explants favoured callus formation on LS medium enriched with 13 µM BAP, 0.5 µM NAA and 20 g/l coconut milk, which differentiated into SLSs. These grew and contained crocin and picrocrocin (Koyama *et al.* 1988). Hori *et al*. (1988) made attempts to induce calli (20-40% frequency) from pistils of saffron on MS, White (White 1963) and Nitsch media enriched with BA and NAA at 1.0 mg/l each. The HPLC analysis from calli revealed the presence of crocin, crocetin- di (monoglucosyl-diglucosyl) ester and crocetin- di (mono-glucosyl) ester. The content ratio of these three pigments in the callus was calculated from the chromatogram in an ODS column as 20: 10: 1 and was the same as in styles. The amount of these pigments in the callus, however was only one tenth of that in the pistils. They could even maintain such calli for more than 2 years in the dark. The authors also suggested that at least a higher level of auxin may be enough for callus induction; more importantly, it is useful to establish cultures having the tendency to differentiate organs for high production of pigments.

Sarma *et al*. (1990) successfully produced SLSs from stigma explants cultured on MS medium fortified with (3 µM BAP) and (54 µM NAA) which contained crocin and picrocrocin, responsible for colour and bitter taste, respectively. However, safranal could not be detected in fresh samples. Fakhrai and Evans (1990) also successfully obtained SLSs from different floral explants but did not observe intense pigmentation from these structures, which is very important. Sarma *et al*. (1991) achieved similar results as reported earlier from ovary explants on MS medium supplemented with (54 µM NAA) and (44 µM BA) but SLSs showed much lower levels of crocin (0.40%) and picrocrocin (0.37%) (6- and 11-fold lower, respectively) than in natural stigmas. Moreover, these authors were the first to publish a report on sensory analysis of spice produced in tissue cultures; this data indicates that saffron pigments produced in tissue cultures were one tenth that of natural stigmas. Further, a sensory profile test showed that tissue-cultured saffron was low in floral, spicy and fatty characteristics which are important characteristics of saffron spice, but was dominant in herbaceous notes-harsh/acrid and barky compared to saffron from natural flowers (Sarma *et al.* 1991). In that study, the % crocin, % picrocin and the crocin/picrocin ratio was 2.4%, 3.9% and 0.6, respectively for stigmas from flowers while the values from tissue-cultured derived stigmas were 0.4%, 0.37% and 1.08, respectively, all values on a dry weight basis. Explants such as shoots and young petals have also been used to regenerate SLSs using 50 µM NAA, 30 µM BAP and 20 g/l coconut milk (Lu *et al.* 1992). Otsuka *et al*. (1992) also induced SLSs from flower, petal or ovary explants on BAP (8, 9-22, 2 µM)- and NAA (0, 5-5, 4 µM)-enriched media with a high level (50-120 g/l) of sucrose in the media. Similarly, Han and Zhang (1993) could also achieve SLSs from other parts

of the flower and subsequently also identified the pigments in these structures.

To increase crocin, picrocrocin and safranal in *in vitro*-grown tissues raised from floral buds on MS medium supplied with 9 µM 2,4-D and 2,3 µM Kn, Visvanath *et al*. (1990, 1994) successfully reported a higher quantity of picrocrocin from callus (obtained from floral buds on MS medium supplemented with 2 mg/l 2,4-D and 0.5 mg/l Kn and filamentous SLSs obtained therein, than in natural stigmas; safranal appeared only in callus but its content was at par with natural stigmas. Crocin content was lower in *in vitro* cultures. A very important observation is that full-strength MS medium supplemented with NAA and BA produced the best caulogenic response (28%) with highest shoot numbers/ovary. On the whole, the best response for shoot growth, both in terms of leaf length and number, was on medium with 0.54 µM NAA and 2.22 µM BA. Ovaries of different growth stages having stigmas of pale yellow, pale orange and bright orange regenerated a maximum mean number (3.8-4.2) of shoots/ovary (Bhagyalakshmi *et al.* 1997; Castellar and Ibora 1997).

An important study on optimization of *in vitro* conditions for frequency of proliferation of SLSs from half-ovary explants of *C. sativus* was carried out by Loskutov *et al*. (1999). The optimum proliferation of SLSs was observed on B5 basal medium (Gamborg *et al*. 1968)) containing 5.4 µM NAA, 44.4 µM BA, MS organics, 0.05% casein hydrolysate and 11.2 µM L-alanine. They reported that the amounts of crocin, crocetin, picrocrocin and safranal in SLSs, as determined by HPLC, were similar to those found in natural saffron stigmas. Guo *et al*. (1999, 2005) used a cell culture technique to produce crocin. Crocin production using *C. sativus* callus by a two-stage culture system was reported by Chen *et al*. (2003). Saffron callus was grown in a two-stage culture on B5 medium supplemented with 300 mg/l casein hydrolysate at 22°C in the dark with 2 mg/l NAA and 1 mg/l BA to give maximum biomass (16 g dry wt/l), and with 2 mg/L IAA (indole-3-acetic acid) and 0.5 mg/l BA for crocin formation. The maximum crocin production (0.43 g/l) was achieved by this two-stage culture method. This two-stage culture system significantly increased crocin production compared to the one-stage system used by others. The main reason for initiating such type of *in vitro* studies is from a commercial point of view so as to produce SLSs or callus with major secondary metabolites whose concentration will not be less than natural stigmas and will be less expensive on a larger scale. To date, there is no report which shows a technique for *in vitro* production of saffron on a commercial scale. Hence there is much scope for the refinement of the existing techniques of saffron production either through micropropagation or through the production of SLS and callus with major secondary metabolites on a larger scale for its use at the commercial level.

Plant growth regulator treatment

A large, well developed corm produces flowers and the first bunch of aerial shoots from the apical bud. The apical bud also gives rise to 1-2 daughter cormels, which are larger in size than those arising from lateral buds. Suppression of the growth of lateral buds helps in harvesting fewer corms of larger size (mostly flowering) from the mother corm than more smaller sized (non-flowering) corms (DeMastro and Ruta 1993). Corms treated with gibberellins before planting decreases the number of sprouting lateral buds, resulting in the formation of fewer daughter corms. The apical daughter corms grow larger and hence more flowers per corm are formed. Moreover, treatment of dry saffron corms with gibberellins plus other PGRs when dormant (June–July) promotes the formation of additional flower buds from undifferentiated meristems, leading to enhanced flower formation and saffron yield (Azizbekova *et al.* 1978; Kabdal and Joshi 1978; Azizbekova *et al.* 1982; Farooq and Kaul 1983; Chrungoo and Farooq 1984, 1989; Azizbekova and Milyaeva 1999) and consequently of saffron itself. Application of gibberallic acid (GA) (0.001-0.01%) or kinetin (Kn) (0.005-0.001%) to corms stimulated growth and formation of additional buds, leading to the formation of more flowers and increased yield of dry stigmas by 130-150% (Azizbe-kova *et al*. 1978). The best results were obtained by soaking corms in July by a single application of GA (100 or 500 mg/corm) to dormant corms as a concentrated microdrop in the apical notch (Azizbekova *et al*. 1982). The effect of gibberellic acid is reported to stimulate starch breakdown in favour of reducing sugars and pentoses (Chrungoo and Farooq 1989, 1991). An increase in plant height, number of leaves/corm and number of daughter corm/mother corm was reported after an overnight dip of mother corms in an aqueous solution (50 ppm) of 2,4-D (Kabdal and Joshi 1978).

Mechanization

Modernisation of saffron cultivation depends on reducing labour costs through mechanisation efforts (Galigani and Pegna 1999). Cost benefit analysis of saffron cultivation in Kashmir ranges from 1:0.69 to 1:1.39, depending on productivity. According to Alam (2007), cost input in saffron cultivation is very high, the labour component accounting for 47% and other inputs 53%. This demands mechanisation for reducing labour costs as well as for removing drudgery, so that educated youth of saffron farmers may continue traditional farming (Alam 2007). Moreover, with increasing labour wages mechanisation has become imperative for profitability and sustainability of this cash crop. Highly sophisticated costly machines may not be affordable to saffron growers, as most of them are small and marginal farmers, but these machines can be made available on a custom hire basis.

Saffron is a difficult crop to mechanize since the plant is small and delicate, and presently no machines capable of totally mechanizing this crop are available (Alam 2007). Traditionally before planting saffron corms, deep ploughing (30 cm) is done using a bullock-drawn plough and planking. This operation can be mechanized using a tractor and matching ploughs and harrows or a power tiller with a ridger plough (Alam 2007). The field is laid out into strips (2 m wide and 10-20 m long) across the field slope with 30-cm wide and 15-cm deep drainage channels on both sides, to avoid water stagnation to which saffron is sensitive. After bed formation sowing is done by hand dropping saffron corms behind the plough. This operation too can be mechanized using a bed planter or semi-automatic vegetable transplanter (Alam 2007). In trials conducted in Italy with a ridger alone or in combination with a potato planter it was possible to make ridges about 15 cm high and 1 m wide separated by 30-cm furrows (Galigani 1987). Corm planting too can be mechanised using an onion or tulip planter (**Fig. 1D**). However, the major drawback in mechanical depositing of corms in the bed is the lack of consideration to their polarity, and corms planted deviant from their vertical axis or upside down show delayed sprouting and declined productivity (Galigani 1982). Another type of machine that can be adapted to saffron planting is the potato planter. Overall, this machine was found to give a lower yield than the onion planter but provided better control in terms of corm orienttation (Galigani 1987; Galigani and Adamo 1987; Tammaro 1990). It could also be combined with a ridger to prepare and plant in a single operation, reducing the working time (Amato *et al.* 1989). In Italy, trials were conducted in the 1980s by burying zinc-mesh cages with a U cross-section containing the corms, and the cages were expected to last for 3 years (Galigani 1987). Similarly, corms were planted in Spain in a mesh cage (45 cm wide) on raised beds (1.5 m wide) with the help of a tulip planter, in order to facilitate subsequent extraction of corms at the end of the planting cycle. Although this system provides ease to the operator, due to the tendency of the cage to wrap and corms to slide inside the cage, it causes unevenness in planting density (Alam 2007). Another machine for saffron corm planting has been developed (Mohammad 2006).

Weeding and hoeing accounts for the major labour cost component (35% of total labour cost). Several hoeing operations are carried out using locally made rakers with wooden or iron teeth (**Fig. 1E**) to aerate the saffron beds and destroy weeds. A wheel hoe specially designed for clay loam soils can be effectively used for light weeding and hoeing. Besides this, hoeing can also be effectively performed using a two-wheel tractor, taking care not to till at a depth greater than 2-3 cm because of the tendency of new corms to grow close to the surface (Alam 2007). Several companies have designed harrows with back teeth that can be quickly lever-adjusted to different positions to give the desired penetration and vibration action for superb weeding, seedbed preparation, residue incorporation and trash clearing (**Fig. 1F**).

Mechanization of the harvesting in saffron is extremely difficult owing to three main factors, viz. (i) flowers are delicate and grow close to the soil surface, (ii) flowers are usually accompanied by leaves, (iii) the quality of saffron becomes adversely affected if soil clods get picked together with flowers. Hence, mechanical harvesting of flowers would damage foliage and drastically reduce the production of replacement corms (Alam 2007). Garvi (1987) outlined a proposal for the automation of stigma separation in a Spanish patent named "saffron combine harvester" but the whole description was vague, and incomplete. Efforts have been made to separate the styles from the stamens and petals by means of a wind tunnel or a fan with certain modifications (Skrubis 1990). A new machine for automated cutting of saffron flowers to obtain their stigmas has recently been developed (Gracia *et al.* 2009). This new machine has been patented (Gracia *et al.* 2008) and a prototype has been constructed for experimentation and validation. The key point of the invention is the use of a vision system to obtain, using image analysis, the optimal cutting point. Importantly, the proposed automated cutting machine can cut 8,000 flowers h^{-1} under the supervision of one operator, whereas one person only averages about 1000 flowers h^{-1} using the traditional hand method. Therefore the production rate is increased eight times.

In Kashmir, drying is generally carried under shade which takes 27-53 hrs to dry the product to a moisture level of 8-10%, and causes deterioration in its quality. The design and development of equipment for the mechanisation of post-harvest treatment of saffron stigmas in India has been presented by Sama *et al.* (2000) and Anwar (2007). Low-cost solar heated dryers have been designed and fabricated in Kashmir, and these have reduced the drying time to 3-4 hrs and maintained quality (Kamili and Nehvi 2005). A hot air dryer and its modified version have also been designed, especially for inclement weather so that farmers can use it indoors. It is a tray dryer in which heated air (45 ± 5°C) with supplemental heating and operated on electricity and liquefied petroleum gas (Alam 2007).

At the end of the planting cycle saffron corms are dug out from the field. On average, 80-man days/ha are required for digging and gathering saffron corms. Several machines like a groundnut or potato digger can be used with slight adjustments (Alam 2007). A potato-picking machine drawn by a two-wheel drive tractor weighing 270 kg has been successfully used for such an operation (Galigani 1989, Amato *et al.* 1989).

Government support

The saffron industry in Kashmir has been showing a steady decline, particularly during the last decade (Anonymous 2010). Analysis of factors responsible for this primarily includes reduction in production and productivity of this important commercial crop due to corm rot, cultivation under rainfed conditions and lack of adequate nutritional support. In addition, severe drought and scanty rainfall (prior to generative phase of saffron) from 1999-2003 has significantly contributed in the dismal performance of the saffron crop in the last decade.

1. Production and productivity

During the 1990's, a comprehensive research programme for addressing R&D in saffron was launched by Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-K) with financial support by the Indian Council of Agricultural Research under National Agricultural Technology Project (NATP). Based on the researches technological up gradation and refinement resulted in a cost benefit ratio of 1:1.39 (Nehvi 2004). Research under NATP led to standardization of irrigation scheme, fertigation schedule, corm-planting population, disease control, and drying procedure for better saffron yield. The dissemination of these technologies together with support from the Government to promote their adoption by saffron growers should increase saffron production from the current level of 5 t to 20 t in the next few years. Keeping this objective in view, the Government of India launched a project in 2006 under the Technology Mission for Integrated Development of Horticulture, including saffron, under Mini Mission II so that improved technologies could be transferred to farmers' fields and adequate support be provided to facilitate their implementation. The assistance is meant for Integrated Development of Saffron in J&K by creation of an irrigation source at target sites, rejuvenation of old and unproductive saffron fields, creation of vermicompost units and provision of solar dryers to saffron growers. In addition, a National Mission for Kashmiri saffron with financial support of Rs. 37.5 million for irrigation, research, mechanization, processing and marketing support has been announced in 2010 by the Prime Minister of India. Initiatives have been taken for skill up-grading of saffron growers through hands-on training conducted through the Directorate of Extension Education (SKUAST-K) and State Agricultural Management & Extension Training Institute (SAMETI) with regard to improved technologies.

A separate scheme by the Government of India has been recently taken up in order to promote the cultivation of saffron crop in a non-traditional area (Kishtwar district) (Anonymous 2009). This scheme includes provision for providing special training to saffron cultivators, seed distribution, demonstration plots and subsidized tools and kits.

2. Marketing and quality control

Even though the suggestion of local saffron growers for imposition of import duties on saffron from Iran and other countries to ease the competition for Kashmiri saffron remains to be evaluated by the government, Kashmir's saffron sector is poised for serious trouble until smuggling of saffron into the country is effectively plugged. For this, legislations and their enforcement should be as stringent as for smuggling of drugs like heroine, etc. The other important area is the quality-based price evaluation and branding. There is a need for strict enforcement of quality standards by the concerned government agencies. A step in this direction is the recent decision by the government of J&K to set up a packaging unit in the traditional saffron growing area (Pampore) certifying quality under the brand name "*Kungposh*" meaning 'saffron flower'.

One positive aspect of the saffron sector in Kashmir is that the farmers and their associations are now very keen in restoring the credibility of the trade. Their latest move of registering farmer groups as societies would go a long way in bringing in consistency in processing and marketing of Kashmir saffron. In France, for instance, associations of saffron farmers ("*Les safraniers du Gâtinais*" in 1987, and "*Les Safraniers du Quercy*" in 1999) were registered after decades of abandon of the crop for its revival (Fernández 2007).

In view of the rampant adulteration, the government of J&K has proposed some amendments in the Saffron Act for addressing the problems of adulteration and falsification of origin. Once these amendments are passed as law these will surely curb the malpractices to a large extent. However,

there is hardly a substitute to granting the status of Geographical Indication (GI) for Kashmiri saffron when it comes to ensuring its quality standards and market credibility. In Spain the designation of 'protected origin and protected geographical indications' was awarded to the "*Azafrán de la Mancha*" in the beginning of this decade (Commission Reg. 464/2001, OJ L66, 8.3.2001, p. 29), and two years earlier the Greek "*red saffron*" had also gained the same designnation under the name '*Krokos Kozanis*' (Commission Reg. 378/99 OJ L. 46, 20.2.1999, p. 13). Similarly the European Union has also awarded the designation to the Italian "*Zafferano dell'Aquila*" (Ordoudi and Tsimidou 2004; Fernández 2007).

CONCLUSIONS

Kashmir has the potential of becoming a global leader in the saffron industry through the adoption of a scientific agro-technology, better post-harvest management and proper marketing strategy. Apart from factors related to agronomic production practices and lack of scientific aptitude among small and marginal farmers (discussed in Husaini *et al*. 2010), clandestine import of Iranian saffron and rampant adulteration practices have ruined the saffron market of Kashmir. Inadequate infrastructural facilities and poor quality control measures have shaken consumer confidence and therefore need to be addressed in earnest. Iran has set an example for Kashmir to follow. Replacement of marketing systems by a new intervention have made it possible for Iran to export about 120 t of saffron compared to 1.3 t exported from India (2005-06) where farmers are dependent on middlemen, traditional sun drying and family labour. Currently this golden spice is highly remunerative in the world market and therefore offers ample scope for employment generation in Kashmir.

Technological intervention and adoption of novel scientific methods based on breeding and biotechnology can help to address the problem of supplying disease-free cormlets in large numbers at reduced costs. However, these techniques are still not commercially viable and therefore, further research is needed in this direction. In addition, mechanization, though strongly advocated, has not been successful due to the delicacy of certain operations in saffron cultivation and processing. Moreover because of the marginal nature of this crop, investments by manufacturers are unlikely to be repaid as quickly as they would expect. In fact, this is a general limitation with the saffron crop and Kashmir is no exception. However, with a recent increase in the world price of saffron, farmers may be ready to pay for higher costs of machinery to the manufacturers, who may readily invest in designing better machines. A higher international price would also increase its potential of becoming an important source of foreign exchange for India, provided that adequate measures are taken by the political leadership of the country.

ACKNOWLEDGEMENTS

The first author acknowledges the ideas and inputs received from various saffron growers and traders in India, and the support given by officers of the J&K Government.

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How to reference: Author name(s) (2010) Title of chapter. In: Husaini AM (Ed) Saffron. Global Science Books, UK, pp. X-XX

In Vitro Studies on Cormogenesis and Maximization of Corm Size in Saffron

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ABSTRACT

Saffron has long been recognized for its medicinal properties and is used principally as a flavoring and colouring agent in food preparations. The present study assesses the morphogenic response of various explants viz. corm slices, vegetative buds (dormant and active corms) in the formation of cormlets *in vitro*. Maximum cormlets formed on corm slices cultured on half-strength Murashige and Skoog (MS) medium + 6-benzyl amino purine (BAP) 20 µM + α-naphthalene acetic acid (NAA) 20 µM + 30 g/l sucrose. Increase in corm size from active vegetative buds were achieved on half-strength MS medium + indole-3-butyric acid (IBA) 8.8 µM + 40 g/l edible sugar from sugarcane whereas 40 g/l sucrose also favoured such an increase in size with a combination of 2 µM of both BAP + NAA. However, vegetative buds from dormant corms did not grow further or show other responses when cultured with different plant growth regulators or carbohydrate sources due to dormancy.

Keywords: *Crocus sativus*, carbon sources, micropropagation

INTRODUCTION

Saffron (*Crocus sativus*) cultivation in India is mostly confined to the table land of Pampore in Kashmir and Kishtwar in Jammu. The word "saffron" is derived from the Arabic word "zafran", which translates to "yellow" (Husaini *et al*. 2009). Saffron species belong to the family Iridaceae. Its stigmas are dried for use in medicine, food seasoning and colouring since centuries and have the distinction of being the most expensive spice by weight (Dauria *et al*. 2006). Its high cost is primarily due to low yield, labour intensive harvesting and processing of stigma (Nauriyal *et al*. 1977). Saffron contains more than 150 volatile and aroma yielding compounds, the major constituents of commercial importance being crocin (responsible for colour), picrocrocin (flavour) and safranal (smell). It also has many non-volatile active components, many of which are cartenoids including zeaxanthin, lycopene and various α- and β-carotenes. However, saffron's golden yellow-orange colour is primarily the result of α-crocin (Abdullaev 2002).

Major constraints in the production of saffron are non availability of corms (seed material), lack of efforts from farmers or State Government, lack of better cultivation and post harvest practices, rodents and diseases. Since conventional methods of saffron propagation are very slow therefore tissue culture represent an important potential to effectively propagate it (Wani and Mohiddin 2009). Micropropagation of saffron has been advocated as the best alternative for its propagation (George *et al*. 1992; Ahuja *et al*. 1994; Bagheri and Vesal 2006). Reports on *in vitro* corm production (Karaoglu *et al*. 2007; Quadri *et al*. 2008), plantlet regeneration (Majourhat *et al*. 2007), somatic embryogenesis (Raja *et al*. 2007) are indicative of recent attempts in saffron micro propagation. However these have a limited potential for employing in large scale production of corms and therefore an attempt was be made to evaluate morphogenetic response of various explants towards cormlet production and increase the size of mini corms, which is a major bottleneck in the *in vitro* technique.

MATERIALS AND METHODS

Plant material and surface sterilization of explants

Saffron corms (active and dormant) obtained from major saffron-growing sites within the Kashmir Valley served as a source for establishing the *in vitro* cultures. These corms were thoroughly washed with detergent Cedepol (0.5%) and Tween-20 (surfactant) under running tap water followed by final rinsing with double distilled water. Subsequently these were surface disinfected with 70% ethanol for 1 min followed by 0.1% $HgCl_2$ (w/v) for 10 min and washed five times with sterilized double distilled water.

Culture media

Half-strength MS basal medium (Murashige and Skoog 1962) supplemented with different concentrations of sucrose (Qualigens, India) viz. 3, 5, 6, 7, 8 and 10%, 0.8% Difcobacto agar and different concentrations of plant growth regulators (PGRs; Himedia, Mumbai, India) was prepared. All the medium constituents were added together and the pH was adjusted to 5.4 with 1 N NaOH or 1 N HCl and finally dispensed into 100-ml Erlenmyer flasks (borosilicate glass) plugged with non-absorbent cotton prior to autoclaving at 121°C and 15 psi for 20 min. All chemicals and reagents were purchased from Himedia, unless specified otherwise.

Experimental design

Corm slices and vegetative buds were subjected to different treatments as explained below.

Corm slices excised from active corms were cultured on: a) ½-MS medium + 3% sucrose supplemented with 6-benzyl amino purine (BAP) (2–25 µM) + α-naphthalene acetic acid (NAA) (2–20 µM); Kinetin (Kn) 2–25 µM + NAA (2-20 µM). b) ½-MS medium supplemented with 2 µM each of BAP and NAA and varying concentrations of different carbon sources viz. sucrose,

Received: 27 September, 2009. Accepted: 20 November, 2010.

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edible sugar (SLM Sugar, CL Products, India, Ltd.) ranging from 30 to 100 g/l. c) ½-MS medium + 0.5, 1.5 or 2.5 g/l KCl with 2 µM each of BAP and NAA + a carbon source (edible sugar 40 g/l + sucrose 30 g/l).

Vegetative buds from active and dormant corms were cultured separately on: a) ½-MS medium + 2 µM BAP + 2 µM NAA and varying concentrations of sucrose and edible sugar ranging from 30 to 100 g/l. b) ½-MS medium + 8.8 µM indole-3-butyric acid (IBA) + carbon source (edible sugar, sucrose 30-60 g/l each). c) ½-MS medium + KCl (0.5, 1.5 or 2.5 g/l) + BAP (2 µM) + NAA (2 µM) + carbon source (edible sugar 40 g/l + sucrose 30 g/l).

Cultures were maintained in an incubation room at a temp of 25 ± 3°C under a 16-hr photoperiod provided by cool white fluorescent tubes (3000 lux). The experiments were carried out in completely randomized block design (CRD), repeated three times; each treatment had 10 replicates. The size of corms were considered as 'less notable' (LN) when corms weighed < 1.0 g, 'notable' (N; 1.0-1.5 g), and 'prominent' (P; 1.5-2.0 g).

RESULTS AND DISCUSSION

The main aim of the present study was to assess the morphogenetic response of corm slices and explore the possibility for development and standardization of a protocol for multiple cormlet production *in vitro*. Half-strength MS medium supplemented with different concentrations of BAP, Kn and NAA were used to induce *in vitro* cormogenesis from corm slices of saffron (**Table 1**). Corm slices showed a good callus induction response with the combined use of BAP and NAA, with highest callus formation on BAP (20 µM), NAA (20 µM) and 30 g/l sucrose (**Fig. 1A**). However, replacement of BAP with Kn failed to elicit any morphogenetic response. The effect of PGRs is not specific and even different PGRs belonging to the same class may elicit different responses in a given tissue. Most PGRs are rapidly metabolized into physiologically inactive compounds (Bhan 1998) and same appears here with Kn. Callus formation with higher (83-93%) induction frequency was earlier recorded by Ding *et al*. (1981) from corms of saffron using MS medium containing NAA/IAA/2,4–dichloro phenoxyacetic acid (2,4-D) alone at 1 mg/l each. Callus induction has also been reported under the influence of different auxins and cytokinins using different explants saffron. Hori *et al*. (1988) obtained callus from pistils using BAP and NAA (1 mg/l each), while Karamian and Ebrahimzadeh (2001) induced callus from shoot meristems using Kn and 2,4-D. Raja *et al*. (2007) reported callus formation from basal leaf segments using 4.0 mg/l BAP and 0.5 mg/l NAA while Quadri *et al*. (2008) obtained callus from corm slices and needle segments using 13.2 µM BAP, 10 µM Kn, 15 µM 2,4-D and 80 g/l sucrose.

Callus cultures after 12 weeks induced mini-cormlets and most multiple cormlets (20 ± 0.4) were recorded on BAP (20 µM), NAA (20 µM) and 30 g/l sucrose (**Fig. 1B**). A similar response was noted by Quadri *et al*. (2008) with 26.4 µM BAP and 30 g/l sucrose from sub-cultured callus raised from corm slices. Auxin-cytokinin interactions change the course of morphogenetic processes in cultured explants which perhaps caused a shift in the endogenous levels of auxins and cytokinins (Bhan 1998), and in the present study BAP in combination with NAA resulted in reasonably good (20 ± 0.4) mini-cormlet production. *In*

Table 1 *In vitro* response of corm slices of *Crocus sativus* L. on ½-MS medium supplemented with BAP + NAA and Kn + NAA.

BAP (µM)	Kn (µM)	NAA (µM)	Callus*	Cormlet number** Mean ± S.D.
2	-	2	NR	NR
5	-	5	+	5 ± 0.2
10	-	5	+	10 ± 0.5
10	-	10	+	12 ± 0.3
15	-	10	++	13 ± 0.4
15	-	15	++	15 ± 0.2
20	-	15	++	15.5 ± 0.1
20	-	20	+++	20 ± 0.4
25	-	20	++	18 ± 0.3
-	2	2	NR	NR
-	5	5	NR	NR
-	10	5	NR	NR
-	10	10	NR	NR
-	15	10	NR	NR
-	15	15	NR	NR
-	20	15	NR	NR
-	20	20	NR	NR
-	25	20	NR	NR

*Data scored after 8 weeks of culture period; ** Data scored after 12 weeks of culture period; 10 replicates per treatment. S.D. Standard deviation; NR - no response; + low callus; ++ moderate callus; +++ intense callus.
BAP, 6-benzyl amino purine; Kn, kinetin; NAA, α-naphthalene acetic acid

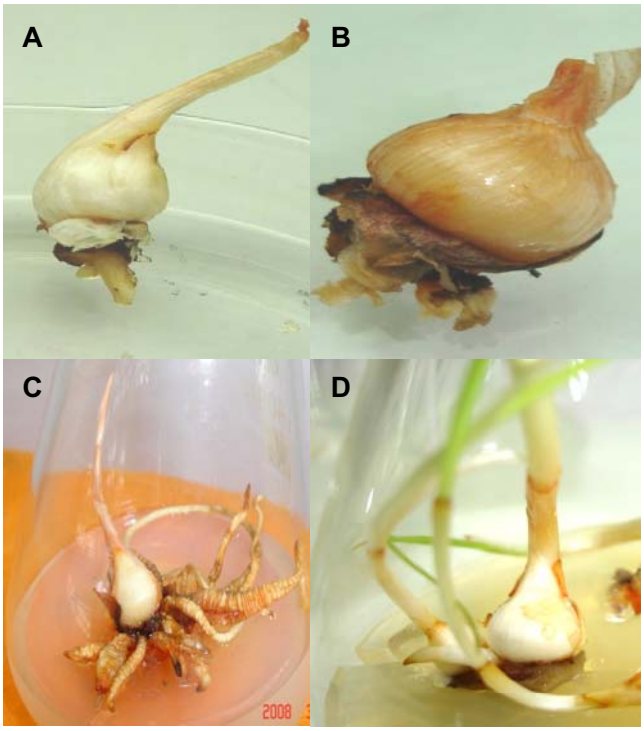

Fig. 2 ***In vitro*** **response of active vegetative buds of** ***Crocus sativus*** **L.** (**A**) Notable increase in corm size on BAP (2 µM) + NAA (2 µM) + 40 and 60 g/l edible sugar after 12 weeks. (**B**) Prominent increase in corm size on BAP (2 µM) + NAA (2 µM) + 40 g/l sucrose after 12 weeks. (**C**) Prominent increase in corm size and multiple thick root formation on IBA (8.8 µM) + 40 g/l edible sugar after 12 weeks. (**D**) Prominent increase in corm size and germination on BAP (2 µM) + NAA (2 µM) + KCl (2.5 g/l) + 40 g/l edible sugar + 30 g/l sucrose after 12 weeks.

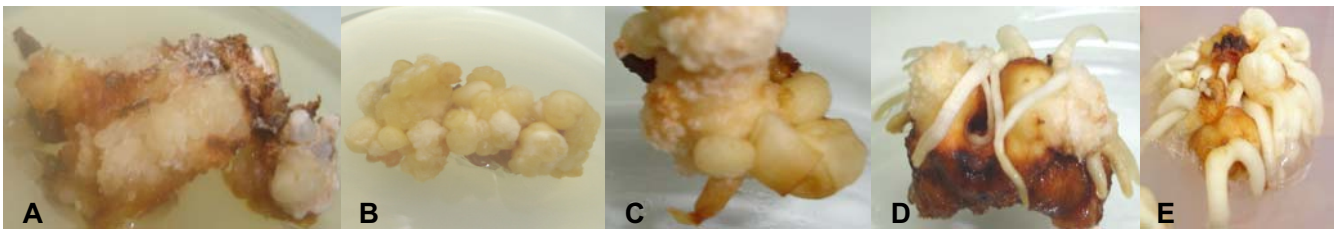

Fig. 1 ***In vitro*** **response of corm slices of** ***Crocus sativus*** **L.** (**A**) Callus formation on corm slices after 8 weeks. (**B**) Multiple cormlet production after 12 weeks. (**C**) Cormlet size (less notable) and callus formation after 12 weeks. (**D**) Cormlet (notable size) accompanied with multiple root regeneration and callus formation after 12 weeks. (**E**) Multiple root formation and increase in cormlet size.

Table 2 Effect of ½-MS medium supplemented with BAP + NAA and different concentrations of carbon sources on size of *in vitro* mini-cormlets of *Crocus sativus* L*.

BAP (µM)	NAA (µM)	Sucrose (g/l)	Edible sugar (g/l)	Corm size	Other responses
2	2	30	-	NR	NR
2	2	-	30	NR	Callus formation
2	2	40	-	NR	Callus formation
2	2	-	40	NR	Callus formation
2	2	60	-	N	Rhizogenesis and callus formation
2	2	-	60	LN	Callus formation
2	2	80	-	NR	Callus formation
2	2	-	80	NR	Callus formation
2	2	100	-	NR	Callus formation
2	2	-	100	NR	Callus formation

*Data scored after 12 weeks of culture period; 10 replicates per treatment. NR - no response; LN - less notable; N - notable.
BAP, 6-benzyl amino purine; NAA, α-naphthalene acetic acid

Table 3 Effect of ½-MS medium supplemented with BAP + NAA and different concentrations of KCl and carbon sources on size of *in vitro* mini-cormlets of *Crocus sativus* L*.

BAP (µM)	NAA (µM)	KCl (g/l)	Sucrose (g/l)	Edible sugar (g/l)	Corm size	Other responses
2	2	0.5	30	40	LN	Rhizogenesis
2	2	1.5	30	40	LN	Rhizogenesis
2	2	2.5	30	40	N	Rhizogenesis

*Data scored after 12 weeks of culture period; 10 replicates per treatment.
BAP, 6-benzyl amino purine; NAA, α-naphthalene acetic acid; KCl, potassium chloride

Table 4 Effect of ½-MS medium supplemented with BAP + NAA and different concentrations of carbon sources on active vegetative buds of *Crocus sativus* L*.

BAP (µM)	NAA (µM)	IBA (µM)	Sucrose (g/l)	Edible sugar (g/l)	Corm size	Other responses
2	2		30	-	LN	-
2	2		-	30	LN	-
2	2		40	-	P	-
2	2		-	40	N	-
2	2		60	-	LN	-
2	2		-	60	N	-
2	2		80	-	NR	-
2	2		-	80	NR	-
2	2		100	-	NR	-
2	2		-	100	NR	-
-	-	8.8	30	-	LN	Rhizogenesis
-	-	8.8	-	30	LN	Rhizogenesis
-	-	8.8	40	-	LN	Rhizogenesis
-	-	8.8	-	40	P	Rhizogenesis
-	-	8.8	60	-	LN	Rhizogenesis
-	-	8.8	-	60	LN	Rhizogenesis

*Data scored after 12 weeks of culture period; 10 replicates per treatment. NR - no response; LN - less notable; N - notable; P - prominent.
BAP, 6-benzyl amino purine; IBA, indole-3-butyric acid; NAA, α-naphthalene acetic acid

vitro mini-corm production has also been obtained by culturing basal leaf segments of saffron on MS medium containing BA (4.0 mg/l) and NAA (0.50 mg/l) (Raja *et al*. 2007). Thidiazuron (TDZ) (0.1 mg/l), however, is much more efficient in producing micro-corms, 60% of regenerants fully developing a leaf primordium compared to only 20% of regenerants with BAP (2 mg/l) (Blazquez *et al*. 2001). This shows that PGRs have an important role to play in cormlet induction.

In another experimental trial *in vitro* raised mini-cormlets and vegetative buds (dormant and active) were assessed for their response to different PGR combinations and carbon sources to enhance their size and regeneration. *In vitro* raised mini-cormlets subcultured on constant BAP + NAA (2 µM each) in ½-MS medium with different concentrations of carbon sources showed a limited response (**Table 2**). A less notable increase in size accompanied with callus formation was registered on edible sugar (60 g/l), while 60 g/l of sucrose resulted in a notable increase in size accompanied with multiple root regeneration and callus formation (**Fig 1C, 1D**). Sucrose plays a vital role for cormlet production in saffron (Sharma *et al.* 2008) but in the present study corm size, rather than cormlet production, could be increased by the use of sucrose and edible sugar as carbon sources (**Table 2**). The addition of KCl (used as an additional potash supplement) resulted in an increase in size followed by rhizogenesis and maximum increase in size and multiple thick root formation on ½-MS, BAP (2 µM), NAA (2 µM), KCl (2.5 g/l), 40 g/l edible sugar and 30 g/l sucrose (**Table 3; Fig. 1E**).

Apical and lateral vegetative buds from actively growing corms were cultured on ½-MS medium with a range of PGRs and carbon sources. Apical vegetative buds showed an increase in corm size which was significantly affected by the carbon source used (**Table 4**). An increase in size was noticed after 12 weeks of culture ranging from LN to P. However, lateral vegetative buds (upper and lower) showed no response at all, which is not consistent with the results of Rajabpoor *et al*. (2007), who reported that the lower portion of corm segments were more responsive than the upper portion. Different responses observed from apical and lateral vegetative buds of the corms can be possibly attributed to polar transportation of PGRs and carbon sources and the existence of a gradient (Cook *et al*. 1993). An increase in corm size was registered when edible sugar (40 and 60 g/l) was added to ½-MS medium supplemented with 2 µM each of BAP and NAA (**Fig. 2A**), while an increase in size was recorded on 40 g/l sucrose (**Fig. 2B; Table 4**). An increase

Table 5 Effect of ½-MS medium supplemented with BAP + NAA and different concentrations of KCl and carbon sources on size of active vegetative buds of *Crocus sativus* L*.

BAP (µM)	NAA (µM)	KCl (g/l)	Sucrose (g/l)	Edible sugar (g/l)	Corm size	Other responses
2	2	0.5	30	40	LN	Germination
2	2	1.5	30	40	LN	Germination
2	2	2.5	30	40	P	Germination

*Data scored after 12 weeks of culture period; 10 replicates per treatment.
BAP, 6-benzyl amino purine; NAA, α-naphthalene acetic acid; KCl, potassium chloride

in corm size accompanied by maximum multiple thick root formation was recorded on 40 g/l edible sugar and 8.8 µM IBA (**Fig. 2C; Table 4**). The addition of 2.5 g/l KCl in medium containing ½-MS, 2 µM BAP, 2 µM NAA, edible sugar (40 g/l) and sucrose (30 g/l), resulting in an increase in the size of corm followed by germination (**Table 5; Fig. 2D**).

Vegetative buds from dormant corms did not express their potential for growth because of deep dormancy. It is not only the concentration of a particular PGR that determines a developmental response, but the cell sensitivity towards it also seems to be important (Trewavas 1998). The variation in responses may therefore be due to the cell sensitivity towards the carbon sources used.

ACKNOWLEDGEMENTS

The work presented here has been carried out under the saffron project funded by the Ministry of Science and Technology, DBT Govt. of India, New Delhi for which first two authors are highly thankful to the funding agency.

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